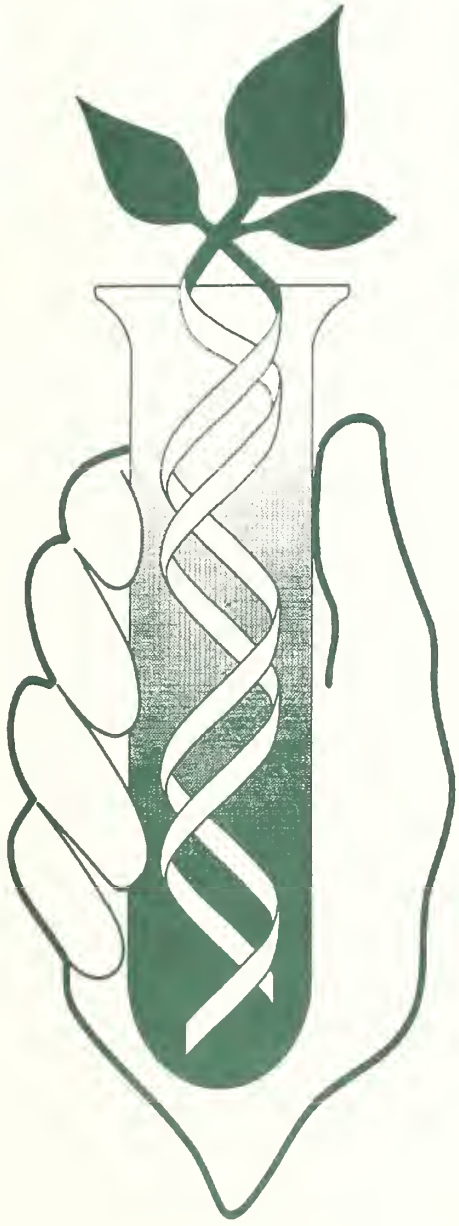

1992

In Vitro Culture and Horticultural Breeding

June 28 - July 2, 1992

Lord Baltimore Hotel
Inner Harbor
Baltimore, Maryland

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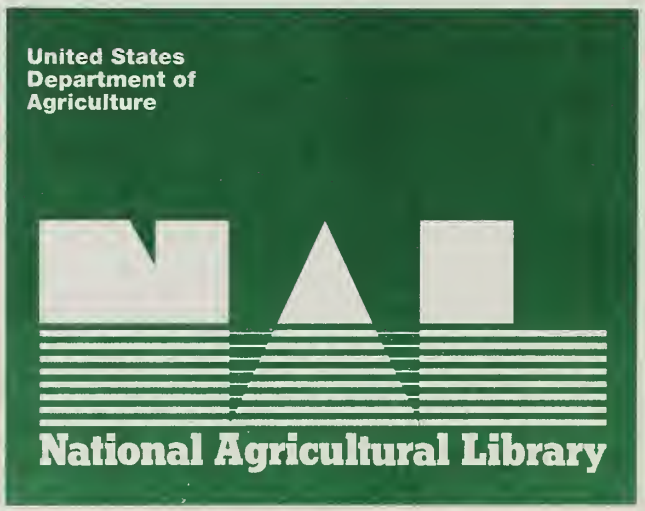

Sunday, June 28

1:00-9:30 Registration

6:30 Welcoming Reception

Monday, June 29

8:30 Welcome:

F.A. Hammerschlag, Symposium Chairperson
Essex Finney, Jr., USDA/ARS, Associate Administrator
M. Faust, ISHS, Fruit Section Co-Chairman

8:45 Keynote Address

Biotechnology and Plant Breeding
F. Bliss

Session I: **Potentially Usefully Horticultural Genes**
Ann Callahan, Moderator

9:30 Lytic peptides and their potential for enhancing plant disease resistance
J.M. Jaynes, L. Destefano-Beltran, P. Nagpala, S.M. Cetiner, and T. Denny*

10:15-10:45 Coffee Break

10:45 Regulation and function of flower senescence-related genes
W.R. Woodson, A. Drory, H. Itzhaki, P.Larsen, J. Maxson, K.Y. Park and H. Wang*

11:05 Isolation of genes affecting peach fruit ripening
A.M. Callahan, R.A. Cohen, L.J. Dunn and P.H. Morgens*

11:25 Development of a saturated linkage map of *Prunus persica* using molecular based marker systems
A. Abbott, L. Beltoff, R. Ballard, W.V. Baird, R. Monet, R. Scorza, P.H. Morgens and A. Callahan*

11:40 Production of genetically engineered color modified chrysanthemum plants carrying a homologous chalcone synthase gene and their field preformance
N. Courtney-Gutterson, E. Firoozabady, C. Lemieux, A. Otten, J. Nicholas, M. Akerboom and K. Robinson*

11:55 Discussion

12:00 Lunch

Posters

-Restriction fragment length polymorphisms in rose and their use for cultivar identification and patent protection
R. Ballard, A. Abbott, S. Rajapakse and J. Kelly*

-Effect of mutagens of peroxidase activity in *Lycopersicon esculentum*
A. Madhu Mohan Rao, K. Srinivasa Charya, S. Karunaker Reddy and S.A. Farooq*

Session II: Strategies for Plant Protection against Biotic and Abiotic Stress
R. Daniel Lineberger, Moderator

1:30 Molecular strategies for the control of plant virus diseases
M. Zaitlin

2:15 *In vitro* methodology for increasing salt tolerance in crop plants
M. Tal

3:00-3:30 Coffee Break

3:30 Perspectives on controlling potato soft rot with foreign genes for antibacterial compounds
S.L. Sinden, R.S. Kobayashi and R.O. Nordeen*

3:50 Opportunities for the use of *in vitro* culture to develop biological strategies through improved production of natural compounds
J.C. Locke

4:10 Coat protein-mediated protection against plum pox virus
M. da Camara Machado, A. da Camara Machado, V. Hanzer, H. Weiss, F. Regner, H. Steinkellner, R. Plail, E. Knapp and H. Katinger*

4:25 Determination of resistance to *Phytophthora cactorum* culture filtrate in apple clonal rootstocks, cultivars and leaf regenerants, using *in vitro* proliferation and optical probe methods
B. Mezzetti, P. Rosati, R.H. Zimmerman and F.A. Hammerschlag*

4:40 Discussion

Posters

-Detection of *Prunus* necrotic ringspot virus serotypes in herbaceous and *Prunus* hosts using a cRNA probe
J.M. Crosslin, R.W. Hammond and F. Hammerschlag*

-*In vitro* selection for *Alternaria alternata* f.sp. *lycopersici* tolerance in tomatoes
A.A. El-Bakry, H.A. Mahfouz, A.M. Tohamy and M.A. Madkour*

-Isolation of *Cucumis melo* mesophyll cells
P. Healey, T.J. Ng and F.A. Hammerschlag*

-Breeding for keeping quality in Christmas begonia (*Begonia* x *cheimantha* Everett) using *in vitro* selection
A.K. Hvoslef-Eide, M. Olsen and T. Field*

-Effects of NaCl and $CaCl_2$ in *Prunus cerasifera* tissue culture: preliminary investigation
*M. Lucchesini and C. Vitagliano**

-The effect of cecropin B on cells and protoplasts of peach
D. Mills and F.A. Hammerschlag*

-Expression of a genetically engineered bacterial disease resistance gene in tobacco plants
R.O. Nordeen, S.L. Sinden, J.P. Kochansky, R. Wagner, J.M. Jaynes and L.D. Owens*

-Evidence of a toxin in *Phytophthora fragariae* culture filtrate
L.J. Rowland, J. Chartisathian, J.L. Maas and G.J. Galletta*

-*In vitro* micropropagation of indicator plants for detecting *Prunus* virus diseases
S. Zilkah, E. Faingersh, A. Rotbaum and A. Stein*

-Transformation of potato (*Solanum tuberosum*) with a gene for an antibacterial protein, cecropin
M. Hassan, R.O. Nordeen, R.S. Kobayashi and S.L. Sinden*

-*In vitro* evaluation of salt tolerance in strawberry (*Fragaria* sp.) seedlings and plantlets
H.G. Hughes, G. Volk and V. Esensee*

7:00-9:00 **Poster Session with Refreshments**

Tuesday, June 30

Session III: **Advances in Transformation and Field Studies of Tissue Cultured and Transgenic Plants**
David James, Moderator

8:30 Field evaluation of tomatoes genetically engineered for enhanced firmness and self life
W.R. Hiatt, R. Sanders, S. Vanderpan, J. Small and M. Kramer*

9:15 One biotech company's solutions to traditional agrichemical problems: or how not to genetically engineer the plant
J.W. Fahey

9:35 Field evaluation of tissue culture-derived peach trees for susceptibility to bacterial spot (*Xanthomonas campestris* pv. *pruni*)
D.F. Ritchie, D.J. Werner and F.A. Hammerschlag*

10:00-10:30 Coffee Break

10:30 Incorporation of the GUS gene into orchids through embryo electrophoresis
R.J. Griesbach

10:50 Transgenic coat protein and antisense RNA resistance to bean yellow mosaic potyvirus
J.J. Hammond and K.K. Kamo*

11:10 Transgenic apples: advances in transformation and field studies of tissue cultured and transgenic plants
D.J. James, A.J. Passey, A.D. Webster, S.L. Uratsu, P. Viss and A.M. Dandekar*

11:25 Comparative field performance of micropropagated red raspberry under two cane management systems
R. Deng, D. Donnelly and D. Buszard*

11:40 Discussion

Posters

-*Agrobacterium rhizogenes* induces rooting in *in vitro* produced shoots of the cactus *Leuchtenbergia principis*
B. Rodriguez-Garay, R. Lopez-Gomez, C. Bayardo-Prieto and E.N. Vivas de la Torre*

-Transient expression of the uid A gene following microprojectile bombardment of plum (*Prunus domestica*) hypocotyls and cotyledons
R. Scorza and J.M. Cordts*

-The MD-VA *Rubus* breeding program
H.J. Swartz, H.D. Stiles, J.A. Fiola and S.K. Naess*

-Direct gene transfer procedures for *Eucalyptus* genetic transformation
C. Teulieres, N. Leborgne and A.M. Boudet*

-*Agrobacterium*-mediated transformation and regeneration of European birch
A.D. Leege, R.R. Tripepi, P.J. Shiel and P.H. Berger*

-Susceptibility of European birch to infection by various strains of *Agrobacterium*
R.R. Tripepi and A.D. Leege*

-Efficient shoot regeneration from leaf sections of highbush blueberry suitable for use in *Agrobacterium*-mediated transformation
L.J. Rowland and E.L. Ogden*

-Regeneration and *Agrobacterium*-mediated transformation of chrysanthemum
L.A. Urban, J.M. Sherman, J.W. Moyer and M. Daub*

-*Agrobacterium*-mediated transformation of apple cv. Red Delicious (*Malus* x *domestica* Borkh.)
S. Sriskandarajah, P.B. Goodwin and J. Speirs*

-Genetic transformation of *Rubus, Ribes, Fragaria* and *Vaccinium*
R.J. McNicol and J. Graham*

-Improvement of *Agrobacterium*-mediated gene transfer of apple (*Malus* x *domestica*)
A. De Bondt, K. Eggermont, M. De Vil, P. Druart, B.P.A. Cammue and W.F. Broekaert*

12:00 Lunch and Tours

Wednesday, July 1

Session IV: **Advances in Somatic Embryogenesis and Organogenesis**
Dennis Gray, Moderator

8:30 Advances in embryogenesis and organogenesis
R.E. Litz

9:15 Agricultural uses of somatic embryos
J. Janick

9:35 The molecular basis for somatic embryo development in carrot
J.L. Zimmerman

10:00-10:30 Coffee Break

10:30 A model system for studying root regeneration in woody species
M. Welander

10:45 Development of nucellar somatic embryos of *Theobroma cacao*
A. Figueira and J. Janick*

11:00 Somatic embryogenesis in sweet potato
A. Sonnino and P. Mini*

11:15 Cacao somatic embryogenesis
M.R. Sondahl, S. Liu and A. Bragin*

11:30 Discussion

Posters

-*In vitro* propagation of *Averrhoa carambola*- a tropical fruit tree
M.N. Amin

-Cell suspension cultures in strawberry, growth characterization and variability
*F. Blando, A. Niglio, A. Frattarelli and C. Damiano**

-Activated charcoal improves the *in vitro* culture of vanilla
M.A. Bustamante and A. Mejia*

-Synchronization of somatic embryogenesis in tea (*Thea sinensis* L.)
B.D. Mohanty

-Micropropagation and *in vitro* production of novelties of commercially attractive endangered cacti
A. Rubluo, B. Rodriguez-Garay, A. Roa and K. Duval*

-Role of explant type and activated charcoal in propagation of date palm by tissue culture
M. Shaheen

-Increasing bud regeneration in an ornamental camellia
A. Tosca and R. Pandolfi*

-Propagation of flood tolerant jackfruit (*Artocarpusy heterophyllus*) by *in vitro* culture
S.K. Roy, M.S. Islam, T. Hossain and A. Hossain*

-The acclimatization of *in vitro* cultured plantlets in the breeding of seedless table grapes (*Vitis vinifera*) in South Africa
I.A. Trautmann and P. Burger*

-Somatic embryogenesis from leaves of *Lisianthus russellianus* Hook.
B. Ruffoni and F. Massabo*

-Cytological and molecular studies on the process underlying commitment in plant somatic embryogenesis
C. Geri, L. Giorgetti, A. Turrini and V. Nuti Ronchi*

-Embryogenesis in different explants of certain induced mutants of *Helianthus annuus* L.
V. Kiranmai and B. Pratibha Devi*

-Organogenesis in single leaf cultures of *Narcissus*
I. Staikidou and C. Selby*

-Successive phases during rooting of microcutting of *Malus*
G.J. de Klerk, J. ter Brugge and M. Keppel*

-Coffee somatic embryogenesis in liquid cultures
M.R. Sondahl and C. Noriega*

-Organogenesis and regeneration of some Andean fruit species
M. Jordan, M. Obando, L. Iturriaga, A. Goreux and J. Velozo*

12:00 Lunch

Session V: **Manipulation of Protoplasts and the Haploid Genome**
Robert Griesbach, Moderator

1:30 Woody plant protoplast technology revisited
S.J. Ochatt

2:15 Citrus scion and rootstock improvement via somatic hybridization
J.W. Grosser

2:35 Haploids for potato genetics and breeding
S. Pelquin

3:00-3:30 Coffee Break

3:30 Ploidy changes in "Mitchell" petunia
K. Kamo and R.J. Griesbach*

3:50 Somatic hybrids and cybrids between *Senecio fuchsii* Gmel. and *Senecio jacobaea* L.
G.R. Wang and H. Binding*

4:10 Discussion

Posters

-Genetic improvement of European pear rootstocks
J.M. Chartier-Hollis and S.J. Ochatt*

-Isolated microspore culture of *Coffea arabica*
*B. Neuenschwander and M. Dufour**

-Towards the somatic hybridization of shrubby and climbing honeysuckles
*D. Georges, L. Decourtye and S.J. Ochatt**

4:30 Meeting of the ISHS Working Group on In Vitro Culture

6:30 **Social Hour**

7:30 **Banquet**

Thursday, July 2

Session VI: **Strategies for the Use of Somaclonal Variation**
P. Rosati, Moderator

8:30 Somaclonal variation: has it proven useful for plant improvement?
R.M. Skirvin

9:15	Disease resistance, cell culture and somatic recombination *P. Larkin*, P.M. Banks, Y.G. Li and L.H. Spindler*
9:35	Somaclonal variation in breeding for disease resistance in tomato *R.W. van den Bulk* and J.J.M. Dons*
10:00-10:30	Coffee Break
10:30	Factors influencing the occurrence of somaclonal variation in micropropagated bananas *O. Reuveni*, Y. Israeli and S. Golobovitz*
10:50	Somaclonal variation in ornamental plants *S. Mohan Jain*
11:05	Generating tetraploid melons from tissue culture *J.W. Adelberg*, W.B. Rhodes and H.T. Skorupska*
11:20	Discussion

Posters

-The response of peach regenerants, cultivars and seedlings to root-knot nematode
G. Hashmi, F. Hammerschlag, R.N. Huettel and L.R. Krusberg*

-Isoenzymatic analysis of somaclonal variation among regenerants from apple rootstock leaf tissue
G. Martelli, I. Greco, B. Mezzetti and P. Rosati*

-Heritable tissue culture induced variation in *Zinnia marylandica*
S.M. Stieve and D.P. Stimart*

-Resistance to leaf blight disease in somaclones of carrot
*L. Dugdale, S. Isaac and H.A. Collin**

-Somaclonal variants of grapevine obtained by somatic embryogenesis
N. Piven, V. Kuksova, V. Volynkin and Y. Gleda*

11:50	Closing Remarks
12:00	Adjourn

ABSTRACTS

Keynote Address:

Biotechnology and Plant Breeding

BIOTECHNOLOGY AND PLANT BREEDING

F.A. Bliss, Department of Pomology, University of California, Davis, CA 95616

Plant breeding is an integrative discipline that involves identification or generation of genetically variable populations, effective selection to produce improved populations or sub-populations (e.g. clones, inbred lines, hybrids), and adequate testing to establish probable utility as a new cultivar. Contributions of biotechnology to plant improvement will be made in one of the following areas; provision of additional (possibly unique) genetic variation, increased efficiency of selection, and reduced time for testing to establish economic utility. Developments such as virus resistance based on expression of viral coat protein genes (i.e., increased, unique variation), molecular marker-aided selection (improved selection efficiency), and manipulation of major genes responsible for a large portion of total quantitative variation of economic traits will be discussed. The integration of biotechnology into the framework of plant breeding offers new opportunities parallel to those realized from the use of Biometrics to improve parameter estimation and selection efficiency, in the early part of this century.

Session I:

Potentially Usefully Horticultural Genes

LYTIC PEPTIDES AND THEIR POTENTIAL FOR ENHANCING PLANT DISEASE RESISTANCE

J.M. Jaynes*, L. Destefano-Beltran[1], P. Nagpala[2], S.M. Cetiner[3], and T. Denny[4], Department of Biochemistry, Louisiana State University, Baton Rouge, LA 70803, USA., [1]Laboratorium Genetika, Ruksuniversiteit-Gent, Gent, Belgie, [2]Upjohn Corporation, Kalamazoo, MI 49001, USA., [3]Department of Horticulture, Cukurova University, Adana, Turkey, [4]Department of Plant Pathology, University of Georgia, Athens, GA 30602, USA.

Over the last several years, we have been interested in the possibility of inserting existing genes of potential agronomic significance, no matter their source, as a means to improve plant disease resistance. Our initial focus was concentrated on the so-called "lytic peptides" found in insects (primarily Lepidoptera and Diptera). These small proteins possess significant bactericidal activity. The cecropins are the most potent of these antibacterial factors and are present under inducible conditions in *Hyalophora cecropia*. We have made significant progess in extending the range of bioactivities of this and other naturally occurring lytic peptides by designing new synthetic ones utilizing strict structure/function criteria. We now have peptides which possess a remarkable array of activities. In addition to enhanced bactericidal activity, they also possess marked fungicidal and nematocidal activities as well. Genes encoding some of these peptides have been constructed and inserted into several plant species. Initial pathogen challenge results are encouraging and indicate that it may be possible in the not-too-distant-future to produce plants with significantly enhanced disease resistance, using our lytic peptide constructs.

REGULATION AND FUNCTION OF FLOWER SENESCENCE-RELATED GENES

William R. Woodson*, Amir Drory, Hanan Itzhaki, Paul Larsen, Julie Maxson, Ky Young Park and Hong Wang, Department of Horticulture, Purdue University, West Lafayette, IN 47907 USA.

The senescence of carnation (*Dianthus caryophyllus* L.) flower petals is associated with increased synthesis of the phytohormone ethylene. This ethylene serves to initiate and regulate the processes of programmed cell death. We are using molecular approaches to study the mechanisms involved in the induction of ethylene biosynthesis and how ethylene initiates this developmental program. We have isolated and cloned mRNAs which encode the ethylene biosynthetic pathway enzymes 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase. These cDNAs have been used to study the regulation of ethylene biosynthesis in various floral organs during postharvest maturation and following pollination. The increase in ethylene production and onset of petal senescence are associated with dramatic changes in gene expression. Several senescence-related (SR) mRNAs have been isolated and used to study specific changes in gene expression during flower senescence. In several cases, the increase in SR mRNA abundance occurs concomitant with the ethylene climacteric. Indeed, treatment of flowers with inhibitors of ethylene synthesis or action prevents petal senescence and the accumulation of SR mRNAs. Sequence analysis of SR mRNAs has revealed homologies with other known proteins, which in some cases points to putative roles of these genes in regulation of petal senescence. In order to elucidate the molecular mechanisms involved in the regulation of SR gene expression, we have analyzed the *cis*-elements responsible for developmental and hormonal gene regulation during floral senescence. Analysis of expression of chimeric SR-GUS genes following particle bombardment and in stably transformed plants will be presented.

ISOLATION OF GENES AFFECTING PEACH FRUIT RIPENING

A.M. Callahan*, R.A. Cohen, L.J. Dunn, and P.H. Morgens, USDA-ARS, Appalachian Fruit Research Station, Kearneysville, WV 25430, USA.

We are using the techniques of genetic engineering to modify peach fruit in a horticulturally desirable way. One goal is to insert an antisense construction of a gene that affects softening, in order to slow the rate of fruit softening. This may allow the fruit to be harvested in a more mature state while still retaining adequate shelf-life. .One problem is to identify and isolate genes which would be appropriate to modify. We isolated a collection of cDNA clones that represent genes whose level of mRNA is regulated during fruit ripening. These were isolated by differential screens using RNA from various stages of softening and RNA from phenotypically different fruit. In addition, several clones were isolated using homology to genes known to be involved in the ripening process.

To determine which genes would be most appropriate to manipulate, three sets of experiments were performed: 1) The time in fruit development at which the corresponding mRNA accumulated. 2) Whether this level of accumulation was associated with defined traits such as softening or texture. 3) The clones were sequenced to attempt to identify their product by comparison to other sequenced genes. The clones fell into four patterns of RNA accumulation: decrease during fruit development; increase during embryo development; increase during ripening; and an increase that correlated with the narrower window of softening. When accumulation was examined in cultivars with different phenotypes, the patterns or amounts of RNA accumulation did not correlate with phenotype. One exception was that none of the ripening and softening associated RNAs accumulated to the same extent in a peach-almond hybrid whose fruit were almond-like with a firm, non-softening mesocarp. Sequencing resulted in the tentative identification of one clone as ACC oxidase or the ethylene forming enzyme. These genes are being modified in many systems to retard ethylene production. In fruit, decreasing ethylene may slow down the ripening process hence extending the softening period. This gene is our first choice for manipulation through antisense constructs for peach.

DEVELOPMENT OF A SATURATED LINKAGE MAP OF *PRUNUS PERSICA* USING MOLECULAR BASED MARKER SYSTEMS.

A. Abbott*, L. Beltoff, R. Ballard, W.V. Baird[1], R. Monet[2], R. Scorza[3], P. Morgens[3], and A. Callahan[3]
Department of Biological Sciences, Clemson University, SC 29634, [1]Department of Horticulture, Clemson University, SC 29634, USA; [2]INRA, Centres de Reserches de Bordeaux, France; [3]Appalachian Fruit Research Station, Kearneysville, WV 25430, USA.

The Rosaceae is a large and economically important family. Besides roses and other species of ornamental significance it includes many of our temperate fruit crops (e.g., peaches, apples, pears and berries). Stone fruits represent an important segment within this family. These include, peach (and nectarine), almond, apricot, cherry (sweet and sour), plum and prune within the genus *Prunus*. To provide a tool to effectively manipulate important agronomic characters in *Prunus* species, we are developing a molecular genetic map of peach using RFLP and RAPD markers. For this purpose, we have used two sets of crosses in peach; an F1 population derived from a cross between the cultivars Jalhousie and Summergrand and four families derived from selfs of four F1 progeny of a cross between cultivars 77119 and New Jersey Pillar. In the first cross we have scored 11 linkage groups with linkage between one morphological marker (flat peach) and an RFLP locus. In the other cross, we have defined 15 linkage groups. Currently we are adding additional markers through further screening of random genomic, cDNA clones, and RAPD markers. With additional markers, we hope to combine our linkage groups till we obtain 8 linkage groups. Using flow cytotometry, we have estimated the peach genome size to be in the order of $6X10^8$ bps. Thus we should be able to obtain a saturated map with relatively few markers in comparison with those maps in other plant species. Our goal is to link molecular markers with genes for resistance to biotic and abiotic factors (disease resistance, cold hardiness).

PRODUCTION OF GENETICALLY ENGINEERED COLOR MODIFIED CHRYSANTHEMUM PLANTS CARRYING HOMOLOGOUS CHALCONE SYNTHASE GENE AND THEIR FIELD PERFORMANCE.

N. Courtney-Gutterson, E. Firoozabady*, C. Lemieux, A. Otten+ J. Nicholas, M. Akerboom+, and K. Robinson, DNA Plant Technology Corporation, 6701 San Pablo Ave., Oakland, CA 94608, USA, and +Florigene BV, Waardlaan 4a, NL-2231 NA, Rijnsburg, The Netherlands.

We introduced a chimeric chalcone synthase (CHS) gene isolated from chrysanthemum into cv. Moneymaker (a pink decorative anemone-centered type), via Agrobacterium-mediated leaf disk transformation, to produce white-flowered plants. The CHS coding sequence was in either antisense or sense orientation relative to the CaMV 35S promoter. 3.6% (3/83) antisense-transformed plants and 1.5% (2/133) sense-transformed plants produced completely white flowers. Pigment analysis revealed that this is due to a block at CHS. To study stability of color change and horticultural characteristics of the white Moneymaker plants, field trials were carried out. Four lines were compared: Moneymaker, a Moneymaker regenerant, an antisense white (2706), and a sense white (31435). There was no difference between Moneymaker and the regenerant. Both 2706 and 31435 were vegetatively propagated with good stability; all plants produced white or very pale pink flowers. The proportion of plants producing pale pink flowers varied among field trial locations. Both 2706 and 31435 flowered later than the control plants, with 2706 about 7 days late and 31435 about 10-12 days late. Flower number was similar for all four lines tested.

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN ROSE AND THEIR USE FOR CULTIVAR IDENTIFICATION AND PATENT PROTECTION

R Ballard*, A. Abbott, S. Rajapakse[1], and J. Kelly[1]. Department of Biological Sciences, Clemson University, SC 29634, [1]Department of Horticulture, Clemson University, SC 29634, USA.

We have cloned restriction fragment length polymorphism (RFLP) probes that can be used as genetic markers for identification, certification, or patent protection of rose. Random genomic clones were generated by shotgun cloning *Hin*dIII fragments of DNA isolated from the rose cultivar 'Confection'. Individual clones were screened by Southern hybridization methods; those clones that have displayed polymorphisms are being used to obtain an RFLP profile, i.e., a DNA fingerprint, for each cultivar. We have identified 15 genomic probes that display RFLPs useful in cultivar identification. Two of these probes, pH1.3C and pH1.0C, can individually identify 12 and 11 different cultivars, respectively. A total of 16 cultivars have been distinguished from one another.

EFFECT OF MUTAGENS OF PEROXIDASE ACTIVITY ON LYCOPERSICON ESCULENTUM

A. Madhu Mohan Rao*, K. Srinivasa Charya, S. Karunakar Reddy and S.A. Farooq, Cytogenetics Lab, Department of Botany, Osmania University, Hyderabad 500 007, Andhra Pradesh, India.

Various types of mutants affecting plant morphology were isolated after treatment of Lycopersicon with x-rays, gamma rays, EMS, HA and Colchicine. Mutants such as dwarf, sterile, reduced number of branches, tall, bushy, dark green and fruit mutants were subjected to the study of peroxidase activity in two cultivars. The mutants which deviated in their total peroxidase activity were subjected to polyacrylamide disc electrophoresis to study the isozyme patterns. The dwarf leafy and sterile mutants showed a significant variation in their banding pattern. There was a significant increase in peroxidase activities of the mutants isolated from different treatments. Electrophorograms of the control showed two anodic bands in Lycopersicon, whereas the mutants isolated after different treatments exhibited 2 to 4 bands.

Session II:

Strategies for Plant Protection against Biotic and Abiotic Stress

MOLECULAR STRATEGIES FOR THE CONTROL OF PLANT VIRUS DISEASES

Milton Zaitlin*, Department of Plant Pathology, Cornell University, Ithaca, NY 14853, USA

Since 1985 several strategies employing transformation technology have been developed which enable the introduction into plants of genes conferring virus resistance. By far the most advanced and widely adopted strategy has been that of coat protein-mediated resistance (Powell-Abel *et al.*, *Science* 232:738-743, 1986). To date, this method has been employed with at least 20 viruses representing 14 taxonomic groups. Plants containing virus coat protein genes normally show a delay in symptom production, although immunity to disease is sometimes obtained. Once regulatory constraints are overcome, this technology will undoubtedly result in commercial products. Other plant transformation strategies currently under investigation employ antisense and ribozyme technology, virus-interfering proteins, the use of satellite and defective-interfering RNAs, and the generation of antiviral antibodies in transgenic plants. Although in some cases the results of these latter studies are encouraging, these technologies have yet to be widely adopted for virus disease control. My laboratory has recently developed a new strategy whereby we have transformed plants with non-structural viral gene sequences in order to induce resistance (Golemboski *et al.*, *Proc. Natl. Acad. Sci. USA* 87:6311-6315, 1990). This approach uses sequences from the viral-coded replicase genes. The original work was done with tobacco mosaic virus, but has been extended to cucumber mosaic virus (unpublished). Plants transformed with such sequences are completely immune to virus disease, although with tobacco mosaic virus the initially-inoculated cells were able to support a low level of virus replication (Carr and Zaitlin, *Molecular Plant-Microbe Interactions* 4:579-585, 1991). All of the listed strategies will be discussed.

This work has been supported in part by the Cornell Biotechnology Program and by Grant 90-37262-5293 from the Competitive Grants Program of the United States Department of Agriculture.

IN VITRO METHODOLOGY FOR INCREASING SALT TOLERANCE IN CROP PLANTS

M. Tal*, Department of Life Sciences, Ben Gurion University of the Negev, Beer Sheva 84105, ISRAEL.

Cell and tissue culture techniques, together with conventional breeding and genetic engineering, have been considered as the main three potential genetic approaches for the development of plants with increased tolerance to environmental stresses in general and for salt stress in particular. The ability of growing plant cells like microorganisms, the successful selection of mutant cell lines from cultured cells and the regeneration of whole plants from such cells have stimulated many attempts, most of them during the 1980's, to apply the *in vitro* techniques for the development of salt tolerant plants. The cell culture approach has been proved effective in obtaining salt-tolerant cell lines in many plant species. In almost all of the experiments the salt tolerance of the cells was found to be stable during mitotic divisions in the salt-free media. Unfortunately, although salt-tolerant cell lines are easily selected for, and whole plants were regenerated in about half of the experiments, there have been only a few cases where salt tolerance of the regenerants was determined or its sexual inheritance was verified. A significant proportion of the regenerants have been defective in their development and/or fertility, and from those which were determined, some were found to be salt sensitive. Some of the potential causes for the limited success include : a. lack of correlation between the mechanisms of tolerance operating in cultured cells and those of the whole plant; b. multigenicity of salt tolerance; c. lack of distinction between adapted cells and true mutants. The main possible causes for the limited success and the need for a better understanding of the physiological and genetic mechanisms of salt tolerance in cultured cells and their relationship to salt tolerance of the whole plant are discussed.

PERSPECTIVES ON CONTROLLING POTATO SOFT ROT WITH FOREIGN GENES FOR ANTIBACTERIAL COMPOUNDS.

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Researchers worldwide are attempting to confer bacterial resistance to crop plants by inserting genes for antibacterial polypeptides and proteins. The feasibility of conferring soft rot (*Erwinia carotovora*) resistance to potato tubers by inserting available genes for cecropin or lysozyme was investigated. Cecropin and chicken lysozyme were both lethal to *E. carotovora* at 6-8 $\mu g \cdot ml^{-1}$. Tuber-disk infiltration experiments indicated that lysozyme or cecropin expression levels greater than 200 $\mu g \cdot g^{-1}$ fresh weight tissue in transgenic plants could confer high resistance. Levels less than 3.5 $\mu g \cdot g^{-1}$ had little effect. Cecropin was, however, at least 5-fold more toxic to potato protoplasts suggesting that, of these two antibacterial compounds, lysozyme might be more efficient in conferring resistance. Potential prospects and problems for achieving success with this approach to control of bacterial diseases of plants will be discussed.

OPPORTUNITIES FOR THE USE OF IN VITRO CULTURE TO DEVELOP BIOLOGICAL CONTROL STRATEGIES THROUGH IMPROVED PRODUCTION OF NATURAL PRODUCTS.

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The use of in vitro culture techniques to "clean up" and to propagate "clean stock" is a well established procedure used to eliminate plant pathogens in vegetatively propagated plant materials. However, since many pathogens, as well as insect pests, become a consideration later in the production phase, direct use of in vitro strategies may not be applicable. Since World War II, the control of insect damage and diseases caused by biotic agents has been heavily dependent on the use of synthetic compounds or "pesticides." The advent of increased environmental awareness and concern for worker exposure has caused thinking about pest control strategies to take a significant turn toward "safer" or "softer" materials. The purpose of this presentation is to suggest other possibilities for the use of in vitro culture to expand the horizons of alternative pest and pathogen control.

Natural plant products, such as nicotine and pyrethrin, have long been known to possess pesticidal activity. During the past two decades, researchers have reported a number of "soft" pesticide-like materials derived from the neem tree (Azadirachta indica) which seem to protect plants in benign ways. The quality and quantity of these secondary metabolites vary greatly among biotypes of the neem tree. In vitro culture may provide the means to 1) enhance production of these materials through selection, 2) multiply these selected types, and 3) provide the means to transfer the ability to produce these materials to other plants.

COAT PROTEIN-MEDIATED PROTECTION AGAINST PLUM POX VIRUS

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Leaf explants from Nicotiana clevelandii and Nicotiana benthamiana - herbaceous host plants for PPV - were transformed with Agrobacterium strain LBA 4404 containing the plasmid pBinPPVm. Following the inoculation plants were investigated for development of symptoms and virus accumulation. The phenomenon of virus resistance was observed at different levels when transgenic plants, expressing the coat protein (CP+), and control plants (CP-) were compared after challenge infection with PPV. Nicotiana clevelandii CP+ plants circumvent virus accumulation. After an initial increase in virus titer similar to the control plants, some CP+ plants showed a reduced accumulation of virus and inhibition of the systemic spread, characterized by decrease of the virus titer and formation of new symptomless leaves. In other Nicotiana clevelandii CP+ plants virus accumulation was inhibited and disease symptoms never appeared. Nicotiana benthamiana CP+ plants were also protected. After a temporary virus accumulation, virus titer decreased without the appearance of symptoms with the exception of a few plants, which showed a delay of thirty days in the development of symptoms post challenge infection.

A system was developed which allows the transfer of foreign genes into apricocot cultivars. The marker gene GUS was used for optical evaluation of the efficiency of the transformation system. The coat protein gene of PPV was used to introduce coat protein mediated resistance against one of the most important pathogens of stone fruit trees in Europe and the whole Mediterranean area.

DETERMINATION OF RESISTANCE TO PHYTOPHTHORA CACTORUM CULTURE FILTRATE IN APPLE CLONAL ROOTSTOCKS, CULTIVARS AND LEAF REGENERANTS, USING IN VITRO PROLIFERATION AND OPTICAL PROBE METHODS.

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Several methods have been recently proposed for early screening for disease resistance. The capacity of Phytophthora cactorum (crown rot of apple) culture filtrate (CF) to act as a selective agent was tested on in vitro proliferating apple shoots and on mesophyll cells using an optical probe. With both assays, four rootstocks (M.26, MM.106, MM.111 and Mark), five cultivars (Gala, Liberty, McIntosh, Empire and Jonathan) and several regenerants from rootstocks M.26 and MM.106 have been tested. Both methods were able to characterize differences in tolerance to CF among the rootstocks which correlated with responses in the field. Among cultivars, Gala exhibited the highest tolerance, which was similar to MM.111 (crown rot resistant rootstock). The results obtained with the regenerants in both assays were similar. Increased tolerance to CF was exhibited by one regenerant from M.26. Comparing the results obtained from the two methods, the optical method (monitoring changes in membrane electrical potential) was more effective for clone discrimination and for studies on host-pathogen interaction.

DETECTION OF PRUNUS NECROTIC RINGSPOT VIRUS SEROTYPES IN HERBACEOUS AND *PRUNUS* HOSTS USING A cRNA PROBE

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Complementary DNAs (cDNAs) prepared from genomic RNA of a peach isolate of Prunus necrotic ringspot virus (PNRSV) were used to produce a ^{32}P-labeled complementary RNA (cRNA) probe which was capable of detecting PNRSV in tissue extracts. Labeled RNA transcripts of an 800 bp cDNA fragment inserted into plasmid pGEM-7Zf(+) were obtained using SP6 RNA polymerase. Dot-blot hybridizations of the cRNA probe were compared to ELISA for the detection of PNRSV serotypes in extracts from peach, cherry, and herbaceous hosts. In most tissues the limits of detection of PNRSV were similar by ELISA and cRNA hybridization. However, PNRSV serotype CH30 reacted poorly in ELISA but was readily detected by the cRNA probe. The probe did not detect prune dwarf, apple mosaic, or tobacco streak ilarviruses, or a virus isolated from hops previously considered to be PNRSV. The ability to detect diverse serotypes of PNRSV by utilizing a single cRNA probe should be of value in nursery certification programs, plant quarantines, and in studies of relationships among PNRSV isolates. Additionally, the sequence information obtained from this clone has enabled us to produce primers for PCR amplification of PNRSV genomic sequences. This information will be of use in the long-range research goal to transform peach with the coat protein gene of PNRSV.

IN VITRO SELECTION FOR ALTERNARIA ALTERNATA F.SP. LYCOPERSICI TOLERANCE IN TOMATOES

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Cotyledon explants of two tomato cultivars (Castlerock and Floradado) were subjected to 0.0, 1.0, 3.0, 5.0 and 7.0% concentrations of Alternaria alternata f. sp. lycopersici culture filtrate in their initial culture media. These were Murashige and Skoog basal salt mixture plus Gamborg vitamins and supplemented with: 4.4 μM BA plus 5 μM IAA (medium A), or 4.4 μM BA plus 5 μM kinetin (medium B) or 10 μM zeatin (medium C). Regeneration was achieved on all media on all fungal filtrate concentrations with medium C being the most inductive. Explants were subcultured onto MS supplemented with zeatin and IAA for shoot multiplication and growth. The media contained 3% fungal filtrate. Rooting was successfully achieved on half-strength MS basal salt mixture plus full-strength Gamborg vitamins with 3% fungal filtrate added. Plants were acclimated, transferred to the greenhouse and are being tested for their in vivo tolerance.

ISOLATION OF *CUCUMIS MELO* L. MESOPHYLL CELLS

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Pathogen-induced toxins can be used as in vitro selective agents when it has been determined that the effects of the toxin at the cellular level correlate with the response at the whole plant level. Mesophyll cells are desirable targets for studying responses to pathogens or toxins that induce symptoms on leaves. In order to study host-pathogen interaction at the cellular level, isolated cells must retain viability over time. A protocol for the isolation of viable muskmelon mesophyll cells was developed in order to study the effects of roridin E, a toxin produced by *Myrothecium roridum*, on cells of leaf spot resistant and susceptible muskmelon cultivars. Factors investigated include the concentration of cellulysin and macerase, desalting the cellulysin, digestion time and culture medium . Viability was determined with fluorescein diacetate. Leaf tissue exposed for one hour to 1% cellulysin and 5% macerase in a B_5 medium with 0.4M sucrose yielded the most viable cells. Viability was higher when desalted cellulysin was used. The optical probe Merocyanine 540 (MC-540) was used to monitor changes in transmembrane electrical potential (PD). Membrane stability over time was monitored by measuring changes in the fluorescence of mesophyll cells stained with MC-540.

BREEDING FOR KEEPING QUALITY IN CHRISTMAS BEGONIA (*BEGONIA* X *CHEIMANTHA* EVERETT) USING *IN VITRO* SELECTION

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Christmas begonia is a popular winter pot plant in Scandinavia. It is a species hybrid produced from crossing *B. socotrana* and *B. dregei*, with subsequent backcrossing of the hybrid to *B. socotrana*. The first crossing was performed in France in 1891. The present cultivars are derived from a single sport which arose in 1948, and therefore have a narrow genetic background. The largest problem for Christmas begonias today is the relatively short keeping quality, compared with plants like Poinsettia, its greatest competitor in the Christmas pot plant market. Keeping quality is closely correlated with sensitivity to ethylene. In a large breeding scheme for Christmas begonia, we have developed a selection method to screen large numbers of *in vitro* plants for ethylene sensitivity. Flowering plants of commercial cultivars were exposed to ethylene (160 nl l^{-1}) for 1,3, and 50 days. They were ranked after testing in a simulated interior climate according to their performance for important characters with respect to keeping quality. The same cultivars were multiplied *in vitro* and given an ethylene exposure of 160 nl l^{-1} for 6,8,10 or 12 h under sterile conditions. These cultures were evaluated according to the yellowing of the *in vitro* produced shoots after exposure to ethylene. The results with flowering plants and *in vitro* shoots were similar, the ranking of the cultivars being equal. Thus this method can be used to select the least ethylene sensitive plants amongst thousands of *in vitro* shoots. The breeding program is continuing with induction of mutants *in vitro*, and screening while still *in vitro*. To widen the genetic basis in improving the keeping quality of Christmas begonia, crosses between the original parents species have been made, and the progeny selected for ethylene sensitivity in the greenhouse. The best progeny are then entered into the mutagenesis scheme.

EFFECTS OF NaCl AND $CaCl_2$ IN PRUNUS CERASIFERA TISSUE CULTURE: PRELIMINARY INVESTIGATION

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Induction of salt tolerance in crop species is an important approach for cultivation in high salt soils. Many reports on several species indicate that high concentrations of Ca^{++} in nutrient solutions mitigate the adverse effects of elevated concentrations of NaCl. In our trials, in vitro derived plantlets of Prunus cerasifera were cultured under NaCl-stress conditions at high or low $CaCl_2$ concentration. Specifically, four increasing NaCl concentrations (35, 100, 150, and 250 mM) in combination with 3 and 10 mM $CaCl_2$ were added to the basal culture medium (modified Murashige and Skoog medium). Results obtained with low $CaCl_2$ showed a serious decrease of shoot development on medium containing 250 mM of NaCl. On the contrary, the best development of in vitro shoots was observed at 35 and 100 mM NaCl. In high Ca^{++} concentration, data showed a completely different situation since plantlets on the highest concentration of NaCl (250 mM) had the best shoot development. At the same high Ca^{++} level, the 35 mM NaCl concentration resulted negative for shoot production. These results, presumably due to a reduction of NaCl availability, confirm the role of Ca^{++} as an antagonist of Na^{+}.

THE EFFECT OF CECROPIN B ON CELLS AND PROTOPLASTS OF PEACH

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Pathogenic bacteria, such as *Xanthomonas campestris* pv. *pruni*, cause diseases of significant economic implications in the genus *Prunus*. Cecropins are naturally occurring bactericidal peptides found in the hemolymph of insects. Cecropins cause channel formation in membranes and lysis of bacterial cells. We are interested in engineering the gene for cecropin into peach (*Prunus persica*) and other fruit tree species. The objective of this study was to determine the effect of cecropin B on viability, using fluorescein diacetate staining, and on changes in transmembrane electrical potential (PD) using the fluorescing probe merocyanine-540. Protoplasts were isolated from shoot-tip cultures in a CPW13M (salts + 0.71M mannitol) solution containing 2% cellulase and 0.5% macerase, while cells were isolated in CPW15.4S (salts + 0.45M sucrose) containing 0.5% cellulase and 0.5% macerase. Cells and protoplasts responded similarly to cecropin B. A concentration up to 10µM cecropin B had a slight effect on viability and changes in PD. Greater concentrations caused significant depolarization and decrease in viability. Cecropin B at a concentration of 100µM caused total mortality of cells and protoplasts. These results suggest that cells and protoplasts of peach can resist cecropin B in the concentration range that causes lysis of plant pathogenic bacteria. The implication of using cecropin to increase microbial disease resistance will be discussed.

EXPRESSION OF A GENETICALLY ENGINEERED BACTERIAL DISEASE RESISTANCE GENE IN TOBACCO PLANTS R.O. Nordeen*[1], S.L. Sinden[2], J.P. Kochansky[3], R. Wagner[3], J. M. Jaynes[4], and L.D. Owens[1]; [1]Plant Molecular Biology Lab, [2]Vegetable Lab and [3]Insect Neurobiology and Hormone Lab, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705; [4]Department of Biochemistry Louisiana State University, Baton Rouge, LA 70803, USA.

Cecropins are a family of small basic polypeptides (~4 kD). They possess potent antibacterial activity and are important in the immune response of insects. We have investigated the feasibility of introducing a cecropin gene into plants to protect them from bacterial disease. To investigate host toxicity, the lethal concentration of chemically synthesized cecropin SB37 was determined for protoplasts from eleven plant species. Lethal concentrations were also determined for nine bacterial species pathogenic to these plants. Lethal concentrations ranged from 4.5 µM for tomato (*Lycopersicon esculentum* cv Red Cherry) to 41 µM for sugarbeet (*Beta vulgaris* cv REL 1). Lethal concentrations for the bacteria ranged from 0.3 µM for *Pseudomonas syringae* pv *glycinea* strain Pg4 to 4.5 µM for *P. syringae* pv *tomato* strain 0683-23. A cecropin gene was fused to a plant secretory signal DNA sequence by PCR (polymerase chain reaction) and introduced into tobacco (*Nicotiana tabacum* cv Bright Yellow) by transformation with *A. tumefaciens*. Cecropin derivatives including MB39, the expected gene product of the PCR gene fusion, were chemically synthesized and the lethal concentrations with respect to Escherichia coli strain D21 were examined. The lethal concentration of MB39 was 0.87 µM compared to 0.28 µM for cecropin B, the insect form. The reduced toxicity of MB39 and other synthetic cecropins tested was dependent on alterations at the amino terminus of these proteins. The progeny of transformed plants that were resistant to kanamycin were analyzed for β-glucuronidase and cecropin gene expression. Northern hybridization analysis indicated that about 75% of R_1 plants produced a transcript of the expected size that hybridized specifically with a cecropin gene probe. The stability of the cecropin protein in these transgenic tobacco plants is currently being analyzed. The toxicity studies and transcription analysis indicate it may be possible to obtain bacterial disease resistance in plants utilizing a modified cecropin gene.

IN VITRO MICROPROPAGATION OF INDICATOR PLANTS FOR DETECTING PRUNUS VIRUS DISEASES

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The objective of this study was to develop in vitro indexing for improving fruit tree quarantine procedures. Three plant indicators to Prunus Necrotic Ring Spot Virus (PNRSV) disease were micropropagated: Prunus persica 'Elberta', Prunus tomentosa and Prunus serrulata 'Shirofugen'. The media for shoot culture establishment were Almehdi and Parfitt (AP) containing 1 μM BA and 0.05 μM IBA for both P. tomentosa and 'Elberta' and Boxus containing 5 μM BA, 0.3 μM GA_3 and 0.1 μM 2,4-D for 'Shirofugen'. The media for shoot proliferation were AP + 0.05 μM IBA, 2 μM BA and 0.7 μM GA_3 for both 'Shirofugen' and 'Elberta' and AP + 0.05 μM IBA, and 1 μM BA for P. tomentosa. The rooting medium was half-strength Murashige and Skoog basal medium containing 3 μM NAA for both P. tomentosa and 'Elberta' or 5 μM IBA for 'Shirofugen'. PNRSC infected peach (cv. Hermosa) was reciprocal grafted in sterile conditions on the indicators. Virus transmission to the indicator has been detected by ELISA method. The in vitro conditions are manipulated for increasing the detection efficiency as biological symptoms and serological method as well.

EVIDENCE OF A TOXIN IN PHYTOPHTHORA FRAGARIAE CULTURE FILTRATE

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We have investigated the potential use of Phytophthora fragariae culture filtrate (CF) for in vitro selection of strawberry variants resistant to red stele. CF was first tested for its ability to induce wilting of tomato and strawberry seedlings. CF did induce wilting of tomato seedlings but did not induce wilting of strawberry seedlings. Second, we observed the effect of CF on ion leakage of strawberry root tissue. A slight increase in conductance was observed in the susceptible variety 'Honeoye' but not in the resistant variety 'Allstar'. Third, the effect of CF on shoot or callus regeneration from leaf sections of 4 strawberry varieties was investigated. Use of 10% or more CF resulted in browning of all leaf sections of susceptible varieties 'Honeoye' and 'Marlate'. However, in the presence of 10% CF, approximately 20% of leaf sections of resistant varieties 'Allstar' and 'Guardian' remained green and/or produced calli. And, fourth, the effect of CF on growth of shoot explants was examined. 20-50% CF had an adverse effect on growth of all varieties tested. Thus, our results suggest the presence of a toxin in CF of Phytophthora fragariae and suggest that a CF-based method of screening for resistant strawberry variants is feasible and worth further study.

TRANSFORMATION OF POTATO (*SOLANUM TUBEROSUM*) WITH A GENE FOR AN ANTI-BACTERIAL PROTEIN, CECROPIN.

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The possibility of protecting plants from their bacterial pathogens by inserting foreign genes for antibacterial proteins such as lysozyme and cecropin has been suggested. A gene for an antibacterial protein, cecropin, fused to a plant secretory signal DNA sequence was introduced into two potato cultivars with *Agrobacterium tumefaciens* using a leaf disk (cv. Atlantic) or microtuber transformation procedure (cv. Frito-Lay FL 1607). Four regenerants of 'Atlantic' and 8 of 'FL 1607' exhibiting kanamycin resistance and expressing the GUS reporter gene were obtained and propagated. Tests of tuber disks for resistance of tubers to bacterial soft rot (*Erwinia carotovora*) indicated only minor differences among the tubers from control plants and putative transformants.

IN VITRO EVALUATION OF SALT TOLERANCE IN STRAWBERRY (FRAGARIA SP) SEEDLINGS AND PLANTLETS

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Seedlings and clones (derived from seedlings) from crosses involving Fragaria x ananassa Duchn. cv Douglas (D) and Fern (F) and F. chiloensis (L.) Duchn. (C) were evaluated for relative growth in MS media supplemented with various levels and types of salts. Aseptically germinated seedlings were initially screened on NaCl supplemented media (at 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5% levels wt/vol). Those crosses with F. chiloensis parentage (D x C, F x C) had greater relative growth as compared to F X D or D X F with regard to fresh and dry weights. Similar trends were evident when NaCl as well as KCl were used in other evaluations. Specific clones that were proliferated and maintained in vitro were evaluated using first NaCl and KCl at levels of 0.2, 0.5 and 0.8% and then various salts (NaCl, KCl, Na_2SO_4, $CaCl_2$, K_2SO_4 and $MgSO_4$) at 0.5%. Clones with C parentage again exhibited greater growth and/or survival depending on salt concentration. The media supplemented with SO_4^- had a greater negative influence on clone survival as compared to Cl^-.

Session III:

Advances in Transformation and Field Studies of Tissue Cultured and Transgenic Plants

FIELD EVALUATION OF TOMATOES GENETICALLY ENGINEERED FOR ENHANCED FIRMNESS AND SHELF-LIFE.

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Calgene, Inc. is developing fresh market tomato varieties using genetic engineering to enhance firmness and shelf-life. These varieties can then be harvested and distributed vine-ripe for increased flavor to the consumer. Increased firmness is obtained by regulating ethylene biosynthesis and the rate of ripening. A number of strategies are being tested, and in particular the expression of a bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene to limit the amount of the ethylene precursor, ACC. Enhanced shelf-life is provided by reducing the amount of the pectin-degrading enzyme polygalacturonase (PG) by expression of antisense gene constructs. Product selection and field evaluation with regard to plant morphology, agronomic performance, and fruit quality will be described.

ONE BIOTECH COMPANY'S SOLUTIONS TO TRADITIONAL AGRICHEMICAL PROBLEMS: OR HOW NOT TO GENETICALLY ENGINEER THE PLANT

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Research at Crop Genetics International (CGI), is focused on the creation of novel crop protection products that are effective and compatible with the environment. The company's InStar™ division is developing manufacturing processes for viral insecticides. The company's InCide™ technology is designed to use genetically engineered plant inoculants to protect corn, rice and other major row crops from insects and fungi. The InCide™ delivery systems that are being developed are proprietary plant "vaccine" systems in which seeds are inoculated with genetically engineered microorganisms. These microorganisms, which live and multiply within the growing plant's vascular system, are being engineered to produce biotoxins that protect the plant against targeted insects or fungal diseases. Functioning within the plant, the InCide™ delivery system is not degraded or dispersed by rain, sunlight, wind and other forces that limit the effectiveness of externally applied insecticides and fungicides. CGI's X-tend™ group is focused on developing weed control systems that combine biological and synthetic herbicidal agents. The broad spectrum systems being developed combine chemical herbicides, in amounts significantly lower than are currently applied, with selected naturally occurring bacterial plant pathogens. Applied as a mixture, the low doses of chemical herbicides stress the weeds, allowing the bacterial pathogens to kill the stressed weeds. The company's Kleentek™ division has developed "disease-free" sugarcane seed products using advanced cell culture technologies. Traditionally, sugarcane growers used their own seedcane to replant their crops. However, these stalks or seedcane pass viral, fungal and bacterial diseases directly to the next season's crop. The use of certified Kleentek™ seedcane for planting stock has permitted dramatic yield enhancements.

FIELD EVALUATION OF TISSUE CULTURE-DERIVED PEACH TREES FOR SUSCEPTIBILITY TO BACTERIAL SPOT (*XANTHOMONAS CAMPESTRIS* PV. *PRUNI*).

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Peach trees from plants regenerated from calli of two immature embryos of 'Redhaven' and five immature embryos of 'Sunhigh' were evaluated under field conditions for susceptibility to bacterial spot in North Carolina. Trees of two regenerants (13-9 and 19-1) were obtained by *in vitro* selection for toxin insensitivity. Tissue culture-derived trees were compared with 'Sunhigh' and 'Redhaven' trees produced from axillary shoots by micropropagation and 'Sunhigh' budded onto 'Lovell' rootstock. In late winter 1988, trees were transplanted into the research site. Budded 'Sunhigh' trees were systematically planted in the site to provide initial sources of inoculum. The tissue culture-derived trees were planted among the budded 'Sunhigh' trees in a completely randomized experimental design with each tree being a replicate. Foliar disease was evaluated 1989-1991. Fruit were evaluated in 1990 and 1991. Bacterial spot occurred naturally in the site all three years; being especially severe in 1990 and 1991. Susceptibility of foliage and fruit to bacterial spot of 'Sunhigh' budded onto 'Lovell' and own-rooted 'Sunhigh' micropropagated from axillary shoots did not differ (P=0.05). Own-rooted, micropropagated Redhaven trees were less susceptible than budded or micropropagated 'Sunhigh'. Fruit and foliage of trees derived from 'Redhaven' regenerant #122-1 were significantly (P=0.05) less susceptible to bacterial spot than were parent 'Redhaven' trees. Trees derived from six regenerants of 'Redhaven' embryo #30 were generally more susceptible than were parent 'Redhaven' trees. Foliage of trees micropropagated from five regenerants of 'Sunhigh' embryo #156 did not differ significantly (P=0.05) in susceptibility; however, there were significant (P=0.05) differences in fruit susceptibility. There were differences in susceptibility among the regenerants from the five different 'Sunhigh' embryos. Trees regenerated from toxin-selected cells exhibited less defoliation and less diseased fruit than budded 'Sunhigh' and 'Sunhigh' regenerated from axillary shoots.

INCORPORATION OF THE GUS GENE INTO ORCHIDS THROUGH EMBRYO ELECTROPHORESIS

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The apical meristems of Calanthe orchid embryos were exposed to 1 mg/ml pBI-121 DNA in an electric field. The pBI-121 plasmid contains the GUS marker gene β glucuronidase under the control of the 35S cauliflower mosaic virus promoter. A pipette containing 0.3% agarose and acetate buffer containing the DNA was placed on the one end of the embryo that contained the apical meristem, while the opposite end was in contact with a pipette containing only buffer and agarose. Uptake of the DNA into the meristem was monitored by 4' 6-diamidino-2-phenylindole (DAPI) fluorescence. Optimal uptake occurred after 10 min of electrophoresis at 10 volts and 0.5 milliamps. Under these conditions, 55% of the embryos survived the treatment and 57% of those which survived were transformed as measured by GUS-positive staining. Leaves from 6 month old plants which developed from the transformed embryos expressed specific patterns of GUS staining.

TRANSGENIC COAT PROTEIN AND ANTISENSE RNA RESISTANCE TO BEAN YELLOW MOSAIC POTYVIRUS

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The coat protein (CP) gene of bean yellow mosaic virus (BYMV), and an antisense (AS) construct consisting of the 3′ half of the CP gene, the complete 3′ non-coding region, and a short poly-A tract were separately introduced to Nicotiana benthamiana leaf pieces by Agrobacterium-mediated transformation. Plants were regenerated and screened for the presence of the desired gene using the polymerase chain reaction (PCR) and (for CP plants) ELISA with monoclonal antibodies. Homozygous R2 populations were challenged by inoculation with various concentrations of BYMV and other potyviruses. At least two types of resistance were observed for both CP and AS plants; some transformants exhibited resistance to initial infection, and others became infected but showed resistance to replication or transport of BYMV. Some plants apparently recovered from infection in later-produced leaves, in which no virus could be detected by ELISA or bioassay. Neither BYMV CP nor AS plants showed significant resistance to either pepper mottle virus or turnip mosaic virus, which are distantly related potyviruses. Other transformation methods are being examined for ornamental plants affected by BYMV, and additional chimeric and deleted constructs are being used to examine the mechanisms and specificity of the types of resistance observed.

TRANSGENIC APPLES: ADVANCES IN TRANSFORMATION AND FIELD STUDIES OF TISSUE CULTURED AND TRANSGENIC PLANTS

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We have investigated a number of parameters limiting the regeneration of transgenic apples using *Agrobacterium* - mediated gene transfer. The presence in the virulence induction medium of the plant signal molecule, acetosyringone, already known to induce the transcriptiom of *vir* genes of *Agrobacterium tumefaciens* and of the osmoprotectant glycine betaine have been shown to increase the efficiency of transformation as monitored by fluorimetric determinations of GUS activity in apple leaf discs. We have generated transgenic apple clones (cv. Greensleeves) carrying both marker and reporter genes such as GUS and *nptII* as well as genes for insect resistance. The latter are a gene encoding a cow pea trypsin inhibitor (CpTI) and the gene CryIA(c) from *Bacillus thuringiensis* encoding an intracrystalline protein. Both gene products are toxic to Lepidoptera. In 1992 in the USA, permission to field trial plants carrying CryIA(c) has recently been obtained from APHIS. Field trials in the UK of several hundred individual untransformed clones of apple trees regenerated *in vitro* from leaf discs of Greensleeves have shown that the fruit from such trees show no obvious sign of somaclonal variation after two cropping years.

COMPARATIVE FIELD PERFORMANCE OF MICROPROPAGATED AND CONVENTIONALLY PROPAGATED RED RASPBERRY UNDER TWO CANE MANAGEMENT SYSTEMS

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Field performance of micropropagated (MP) and conventionally propagated (CP) red raspberry (Rubus idaeus L. cv. Comet and Festival) was examined under hedgerow and stool management systems for 3 seasons (1989 to 1991). All MP plants established well after one growing season compared to 58% survival rates for CP plants 45 days after planting. MP plants were more vigorous than CP plants during the first 2 growing seasons as indicated by taller canes, larger number of primocanes and greater dry matter production. Yields of MP 'Festival' in 1990 were 1/2 to 1/3 the yields of commercial plantings in Quebec while yields from CP 'Festival' and MP and CP 'Comet' were negligible. Yields of MP and CP plants were similar in 1991, however, 'Festival' slightly outyielded 'Comet'. Propagation method had no apparent effect on winter hardiness of either cultivar. Cane management systems had no effect on field performance of either MP or CP plants. Cultivar, propagation method or management system did not affect the net CO_2 uptake rates of primocane or floricane leaves. The net CO_2 uptake rates of primocane leaves ranged from 10 to 16 mg $CO_2 dm^{-2} hr^{-1}$ in 1989 and from 9.5 to 20 mg $CO_2 dm^{-2} hr^{-1}$ in 1990, and those of floricane leaves ranged from 8 to 20 mg $CO_2 dm^{-2} hr^{-1}$ in 1990. After 2 growing seasons, the differences in field performance between MP and CP plants disappeared. The results clearly indicate that MP plants are superior to CP plants for nursery propagation and fruit production due to their more consistent establishment and increased vigor.

AGROBACTERIUM RHIZOGENES INDUCES ROOTING IN IN VITRO PRODUCED SHOOTS OF THE CACTUS LEUCHTENBERGIA PRINCIPIS

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Habitat destruction and illegal overcollection have significantly reduced cacti populations. Convential propagation methods have been found to be difficult and time consuming, mainly due to low germination rate and extremely slow growth in some species (highly appreciated as rare ornamental plants). Plant tissue culture techniques have emerged as powerful and effective propagation tools for conservation and commercial production of cacti.

Leuchtenbergia principis is among the most appreciated species by cacti collectors worldwide. Several shoots obtained through in vitro axillary shoot proliferation were inoculated at the base with Agrobacterium rhizogenes strain LBA-9402 (kindly donated by L. Herrera-Estrella, CINVESTAV, Irapuato, Mexico). After an infection period of 5 days, the inoculated shoots were placed on a medium containing growth regulators for shoot proliferation. Roots started to form after 30 days; while non-inoculated shoots did not produce any roots even in a hormone-free medium. To our knowledge, this is the first report in the production of roots in the cactaceae family by A. rhizogenes. Experiments with several cacti species are now in progress, and we believe that this finding opens the opportunity to create chimeric cactus plants through genetic transformation.

TRANSIENT EXPRESSION OF THE *UIDA* GENE FOLLOWING MICROPROJECTILE BOMBARDMENT OF PLUM (*PRUNUS DOMESTICA* L.) HYPOCOTYLS AND COTYLEDONS.

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Hypocotyls and cotyledons from plum seeds stored for 90 days postharvest were explanted onto regeneration medium (Mante et al., 1989. Plant Cell Tissue Organ Culture 19:1-11). At 2 day intervals explants were exposed to microprojectile bombardment following the methods of Ye et al. (in press). Microprojectiles carried an engineered plasmid with the *uidA* gene coding for GUS expression. The X-Gluc assay was used to visualize GUS expression 48 hours after bombardment. High rates of transient expression were observed on both hypocotyls and cotyledons when bombardment followed a short preculture period. Increasing length of preculture decreased rates of transient expression in both hypocotyls and cotyledons. Coupled with the regeneration systems for plum hypocotyls and cotyledons, which we have previously reported, microprojectile bombardment appears to be a feasible approach to plum transformation.

THE MD-NJ-VA RUBUS BREEDING PROGRAM

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Several methods are being used to improve *Rubus*. Numerous polyploid plants have been produced through the use of 5 mM colchicine and in vitro leaf regeneration. Griseofulvin and Co^{60} γ-radiation have been used to produce off-type plants from leaf regeneration. In an effort to produce thornless chimeras, shoots have been regenerated from in vitro thorny/thornless cultivar graft unions. Only one plant out of 208 has hybrid morphology. ß-glucuronidase and chlorsulfuron resistance genes have been transferred to blackberry plants via *Agrobacterium*-mediated transformation and subsequent shoot regeneration on thidiazuron and 10 μg/ml kanamycin. Several model experiments were used to optimize transformation protocol, e.g. shoots coincubated with *Agrobacterium* for 4 days had more galls than those incubated for 1, 2, 6 or 8 days. Kanamycin, at 10 μg/ml, effectively eliminated non-transformed shoot regeneration, without reducing transformed shoot regeneration.

Around 6000 hybrid seedlings per year are distributed among the cooperators. Species of use in the warm Mid-Atlantic region include *R. flosculosis, R. lasiostylus* and *R. pileatus. R. chamaemorus* has been crossed with three species including synthetic tetraploid 'Reveille' and 'Shawnee' blackberry. Embryo rescue has been critical in production of interspecific hybrids.

DIRECT GENE TRANSFER PROCEDURES FOR EUCALYPTUS GENETIC TRANSFORMATION

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Eucalyptus are fast growing trees of particular interest for pulp industry and their improvement through biotechnology is being actively investigated. Polyethylene glycol or electrical treatment of Eucalyptus gunnii protoplasts derived from callus or cell suspension cultures resulted in a transient expression of reporter gene (β glucuronidase). Cell division of these protoplasts can be observed and microcalluses can be obtained. Electrical treatments also allowed a transient expression of the GUS gene in intact cells. That is quite unusual and very interesting because in our hands the cell suspension cultures are able to regenerate calluses with roots and buds. Microprojectile bombardment experiments on primary calluses using a particle gun are in progress. One potential interest of such a procedure is that calluses can regenerate whole plants through organogenesis.

AGROBACTERIUM-MEDIATED TRANSFORMATION AND REGENERATION OF EUROPEAN BIRCH

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Genetic engineering has the potential to overcome many of the difficulties encountered in breeding tree species. European birch (*Betula pendula* Roth) is the most popular landscape birch throughout Canada and the northern half of the U.S., and this species is also an important timber tree in Scandinavian countries. However, European birch is very susceptible to many pests such as bronze birch borer (*Agrilus anxius* Gory), as well as other insects and diseases. The objective of this research was to develop a transformation protocol using *Agrobacterium*-mediated gene transfer and regenerate transgenic plants. Leaf explants were taken from birch microshoots (*in vitro*), incubated on Woody Plant Medium (WPM) supplemented with 22.5 μM benzyladenine for five days, and then co-cultivated with *Agrobacterium tumefaciens* (C58 or LBA 4404). The *A. tumefaciens* C58 contained a potyvirus-derived construct (pPB3-S[2]) that was used as a marker. The *A. tumefaciens* LBA 4404 contained the construct pWTC304 (kindly provided by Dr. H. Whitely, Univ. of Wash.) that contained a coleopteran-specific toxin gene from *Bacillus thuringiensis*. The NPT II gene was also included in both constructs. Kanamycin (Kan) at 30 mg/L was used to select for transformed shoots. Shoots that grew on WPM containing Kan were rooted and grown in a growth chamber. Regenerated plants appeared phenotypically normal. Southern blot analysis from putative transformants revealed that the genes of interest had been integrated into the birch genome. Therefore, European birch can be genetically engineered via *Agrobacterium*-mediated transformation and desirable genes can be incorporated into this valuable species.

SUSCEPTIBILITY OF EUROPEAN BIRCH TO INFECTION BY VARIOUS STRAINS OF *AGROBACTERIUM*

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The use of *in vitro* genetic manipulation to improve pest resistance of European birch (*Betula pendula* Roth) would be of great interest and commercial value since this species is a popular landscape plant in North America and an important timber tree in northern Europe. The goal of this study was to determine the susceptibility of European birch to various strains of *Agrobacterium* so that this birch may be used in an *Agrobacterium*-mediated transformation system. Nine wild-type strains of *Agrobacterium* (A4, B6, B49c/83, C58, E8/73, K27, R1000, S2/73, and W2/73) were tested for their ability to infect potted and *in vitro* birch plants. Stems of 20 microshoots and five potted plants were inoculated with 40-50 μl of 1 x 10^7 colony forming units per ml of each strain. Resulting galls on microshoots were excised, placed on hormone-free Woody Plant Medium and observed for growth. Galls were later analyzed for opines by paper electrophoresis. All strains formed galls on birch stems, with infection rates ranging from 20% to 100% on both microshoots and potted plants. Strains A4 and B49c/83 infected the most plants and induced the most tumor growth. Galls from all strains survived and grew on hormone-free medium, and tumors from seven strains formed shoots or roots as well. Opine was present in gall tissue induced by all strains except E8/73. Results from opine analysis of regenerated shoots arising from the galls will be presented. This research demonstrated that European birch is quite susceptible to infection and transformation by various *Agrobacterium* strains, even though crown gall is rarely reported for this species.

EFFICIENT SHOOT REGENERATION FROM LEAF SECTIONS OF HIGHBUSH BLUEBERRY SUITABLE FOR USE IN AGROBACTERIUM-MEDIATED TRANSFORMATIONS.

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In our studies aimed at producing transgenic blueberry plants, earlier we have shown that highbush blueberry is susceptible to infection by at least 3 commonly used Agrobacterium tumefaciens strains. More recently, we have examined the relative efficiency of shoot regeneration from leaf sections of 3 different highbush varieties and investigated ways of improving the efficiency of shoot regeneration. Effectiveness of tissue culture medium supplemented with cytokinin conjugate zeatin riboside or cytokinin zeatin has been compared to medium supplemented with cytokinin 2iP. Results indicated that zeatin riboside is more effective than either 2iP or zeatin in promoting shoot regeneration and that leaf sections of the variety 'Sunrise' are particularly amenable to shoot regeneration. Furthermore, effect of increasing concentrations of kanamycin (Kan) on inhibition of shoot regeneration has also been determined. Currently, several attempts have been made to introduce the nptII (Kan) gene into blueberry and shoots have been regenerated on medium containing kanamycin. To date, however, results from Southern blot analyses indicate plants have not yet been successfully transformed, and our efforts are continuing.

REGENERATION AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF CHRYSANTHEMUM.

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Regeneration protocols were developed for three cultivars of chrysanthemum: Hekla, Iridon, and Polaris. Shoots were regenerated from leaf explants of cultivars Hekla and Iridon by continuous culture on Murashige and Skoog (MS) medium containing 11.5 µM indoleacetic acid and 1.0 µM benzyladenine. Shoot regeneration from Polaris was accomplished on the same medium, however successful regeneration required transfer of explants with shoot primordia to hormone-free medium and continuous culture in the absense of blue wavelengths of light. All cultivars were rooted on quarter-strength MS medium lacking hormones. Three non-disarmed strains of *Agrobacterium tumefaciens* (ACH5, A281, Chry5) were evaluated for tumor production on the three chrysanthemum cultivars. Chry5 and A281 were significantly more virulent on all three cultivars than was ACH5, averaging 95%, 92%, and 8% tumor formation, respectively. Tumor formation was not significantly affected by cultivar. Cultivar Iridon was successfully transformed with *Agrobacterium* strain EHA105, a disarmed version of A281, containing the vector plasmid pBI121. Stable, kanamycin-resistant plants were obtained and GUS activity confirmed.

AGROBACTERIUM-MEDIATED TRANSFORMATION OF APPLE CV RED DELICIOUS (*MALUS X DOMESTICA BORKH*)

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The introduction of alien genes by transformation offers the potential for the rapid incorporation of new genes into existing apple cultivars. This presentation reports the transformation of the scion cultivar Red Delicious.
Transformed calli and shoots were produced by a leaf-disc transformation-regeneration method. Leaf explants obtained from shoot cultures *in vitro* were inoculated with *Agrobacterium tumefaciens* containing one of the following plasmids pGV3850::1103GUS, pGV3850::1103neo, pLBA4404 x BI121 or pAGLI x KIWI105. After co-cultivating for 6 days, the explants were placed on selection medium containing kanamycin (100 mg/l),cefotaxime (300 mg/l),thiadiazuron (15μM) and naphthalene acetic acid (5.4mM). Callus growth occurred after about 4 weeks of cultivation and shoot primordia formed from these calli after 8-10 weeks. Callus proliferated well in selection medium for over 12 months. Shoots proliferated in selection medium containing benzyladenine (3μM). The rooting frequency of these transformed shoots in kanamycin (100 mg/l) medium was around 60%. Rooted plants were transferred to pots and grown on in a glasshouse. Transformation of plants has been confirmed by histochemical GUS assay, the presence of nopaline synthase,and evidence from Southerns for gene integration .

GENETIC TRANSFORMATION OF RUBUS, RIBES, FRAGARIA AND VACCINIUM

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Regeneration techniques for leaf or stem sections of red raspberry, blackberry, red raspberry x blackberry hybrids, strawberry, blackcurrant and blueberry have been developed to permit the production of whole plants from a single or a few cells. Gene transfer has been achieved, in conjunction with these techniques, using the GUS, GUS:Intron or NPTII marker genes. Transformations have been confirmed by dot blotting, Southern blotting and by GUS assays. Potentially useful genes for virus and pest resistance have also now been incorporated into raspberry x blackberry hybrids, blackberry and strawberry. These include an Arabis Mosaic Virus satellite and coat protein and the cowpea trypsin inhibitor gene for insect resistance. Transformations have been confirmed by molecular techniques and enzyme assays and bioassays are now being carried out to determine if the levels of gene expression are suitable for plant protection.

IMPROVEMENT OF *AGROBACTERIUM*-MEDIATED GENE TRANSFER TO APPLE (*MALUS DOMESTICA*) CV. JONAGOLD.

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We have studied the transfer of an intron-containing ß-glucuronidase (GUS) marker gene from *Agrobacterium tumefaciens* to wounded apple leaf explants. Several factors that intervene in early steps of the transformation process were analysed.

Infection step: Three different strains of *Agrobacterium tumefaciens* were compared. With the succinamopine strain EHA101 highest GUS expression was obtained. Bacterial cell densities varying from 2.5 to $10x10^8$ cells/ml yielded comparable transformation efficiencies. *Agrobacterium* cells from log phase, midlog phase or stationary phase cultures were equally effective.

Precultivation step: Based on a transient expression assay, precultivation of the explants for 4 to 6 days prior to infection was found to be advantagous. However, 4 weeks after inoculation, non-precultivated leaves performed better than precultivated leaves when scored for the presence of transformed calli. The reason for this discrepancy is not understood.

Cocultivation step: Four days of cocultivation was found to be optimal.

Postcultivation step: Three different gelling agents (agar, agarose, gelrite) were compared in combination with varying levels of the selection antibiotic kanamycin (0 to 100 mg/l). Recovery of transformed calli was highest on media containing gelrite and 50 mg/l kanamycin.

Session IV:

Advances in Somatic Embryogenesis and Organogenesis

ADVANCES IN SOMATIC EMBRYOGENESIS AND ORGANOGENESIS

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An important prerequisite for applying biotechnology to horticultural crops is the availability of efficient regeneration protocols, either organogenesis or somatic embryogenesis, from somatic tissues. Although it has been possible to utilize embryonic or seedling material for establishing morphogenic callus of seed-propagated field crops, this does not have utility with woody perennial and many ornamental species, some of which have been vegetatively propagated for many centuries. For several years it has been perceived that the loss of juvenile characteristics in perennial species is a substantial and critical barrier that must be addressed before morphogenesis from somatic tissues can be obtained. Morphogenesis has been shown to be controlled by genetic and epigenetic factors. This has resulted in more sophisticated approaches to problems of regeneration with difficult-to-regenerate species that are not dependent upon rejuvenation of elite germplasm. The importance of interactions among genotype, explant type and precondition (including stage of development) and the composition of the plant growth medium are now accepted to be critical for morphogenesis. Understanding the sequence of events from induction of morphogenic competency (totipotency) of single cells is essential for applying somatic cell genetics to plants. General models for morphogenesis in different horticultural species will be presented. The development of bioreactor systems for continuous plant production and the targeting of morphogenically competent cells for genetic engineering are logically derived from these models.

AGRICULTURAL USES OF SOMATIC EMBRYOS

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Somatic embryogenesis, the process of embryo initiation and development from cells that are not the direct products of gametic fusion, is a natural phenomenon in many species that is usually associated with cells and/or tissues of the nucellus and integuments, megasgameteophyte, embryo, and endosperm. The experimental production of somatic embryogenesis was first reported from secondary phloem cells of carrot reported independently in 1958 by Steward, Mapes, and Smith and by Reinert; embryogenesis from datura microspores was first reported by Guha and Maheshwari in 1964. The ability to induce somatic embryogenesis may be a universal trail whose occurrence depends on the interaction of an appropriate tissue with an appropriate induction stimulus. Embryogenics, the agricultural exploitation of somatic embryogenesis, has been intensively investigated since that time. There are several potential agricultural applications of somatic embryogenesis including rapid clonal propagation (synthetic seeds); freeing plants of virus; crop improvement (gametoclonal variation, production of homozygous lines via androgenesis, regeneration from single cells for transformation and other uses); germplasm preservation; and metabolite production.

THE MOLECULAR BASIS FOR SOMATIC EMBRYO DEVELOPMENT IN CARROT

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The ability to generate embryos in cell culture, through the process of somatic embryogenesis, has been considered to be an attractive model system for identifying genes expressed during early embryogenesis. However, progress has been relative slow and successes have been fragmentary; each laboratory working toward this goal has identified and characterized one or a few such genes. In recent years, we (and others have come to understand that part of the impediment to clone isolation from somatic embryos has resided in the strategy behind the library construction and screening which relied almost exclusively on a comparison of transcripts present in callus cells and somatic embryos.

We have taken a new approach to this problem; we prepared a cDNA library from polysomal mRNA of globular staged embryos of carrot, and screened it with a "subtracted cDNA probe" in which globular cDNA was prehybridized with polysomal mRNA from zygotic seedlings to remove all common sequences. Using this approach, we have been successful in isolating 30 different clones, all of which are enhanced in globular embryos compared to seedlings. Preliminary sequence analysis on all of these different clones shows that in a single screen, we have isolated clones corresponding to many of the embryo clones isolated by the other labs who have worked in this area over the last seven years, as well as at least 24 new clones which have never been described, and which we are now characterizing. These results validate the utility of the screen to identify embryo-enhanced genes, and suggest to us that, at the molecular level, callus cells are not very different from the embryos that develop from them. Indeed, it appears that many callus cells in an embryogenic culture line are actually pre-embryos, and that many of the mRNAs that direct early embryo development are already present.

A MODEL SYSTEM FOR STUDYING ROOT REGENERATION IN WOODY SPECIES

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This project is part of a joint EEC program with the purpose to obtain information about the initial phases of root initiation at the level of gene expression. A model system of thin stem segments, based on shoots produced in vitro of the apple rootstock Jork M9, was used in response to auxin and 100% of the disks produced 5-7 roots in one week. The rooting procedure has been followed by light microscopy as well as SEM and TEM. The first cell divisions occur in the cambial cells and can be seen after 2-3 days on the induction medium. After 4 days, root primordia can be detected. At the CABO Institute, Wageningen a cDNA library has been prepared from induced and none induced stem segments and after differentiell screening 20 clones have been obtained which later on will be used for in situ hybridization.

DEVELOPMENT OF NUCELLAR SOMATIC EMBRYOS OF *THEOBROMA CACAO*

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Somatic embryogenesis in cacao (*Theobroma cacao* L.) derived from immature zygotic embryos has limited value, because the zygote is an untested genotype. Recently, Sondahl (South African patent 88/3078 and abstract at IPBNet meeting in San Jose, Costa Rica, 1991) has reported somatic embryogenesis from nucellus tissue, which is maternal in origin. We have confirmed the induction of nucellar embryony and developed a protocol for conversion of nucellar somatic embryos into seedlings. Ovules (about 12 mm in length) were extracted from immature pods (80 to 120 mm long) and the surrounding mucilaginous testa removed. At this stage the zygotic embryos were in the globular stage and barely visible macroscopically. The basal end of the ovule, containing the zygotic embryo was detached and the liquid endosperm drained away. The remain ovule was dissected into 4 or 5 pieces and explanted on half strength liquid MS media, supplemented with 117 mM sucrose; 10% coconut water; 4 μM 2,4 D; 0.5 μM 2iP; 0.5 g/liter malt extract, and 0.5 g/liter casein hydrolysate, and placed on a gyratory shaker (100 rpm) in the dark for 60 days at 26C with one transfer to the same media after the first 30 days. The callused pieces were transferred to semi-solid half strength MS media supplemented with 117 mM sucrose; 2.5 μM 2iP; 10% coconut water; 0.1 g/liter malt extract; and 0.2% PVP 10,000 for 60 days under light. When recultured on semi-solid half strength MS media, supplemented with 117 mM sucrose; 10% coconut water; and 1 g/liter casein hydrolysate, somatic embryos began to appear at low frequency after 30 days. The embryogenic calli maintained in this medium, developed budding centers (rapidly proliferating globular embryos). Somatic embryos were converted to seedlings using the following procedure: culture in half-strength liquid MS medium supplemented with 234 mM sorbitol and 29 mM sucrose for 15 days, then transfer to semi-solid WPM, with 88 mM fructose, and place in high CO_2 chamber (20,000 ppm) with 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PAR).

SOMATIC EMBRYOGENESIS IN SWEET POTATO

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Mutation induction represents a promising tool for improvement of vegetatively propagated plants, such as sweet potato. Chimerism is an undesired phenomenon occurring after a mutagenic treatment of a multicellular structure such as a seed or a bud meristem. Valuable or desired mutations could be lost during the ontogenesis of individual shoots. Somatic embryogenesis may make it possible to obtain plants which are of single cell origin. Sweet potato plants of five clones (Q 23728, Q 23836, CN 1489-89, 288-6B, W 190 F) were tested for their somatic embryogenesis capacity. Shoot cuttings (20-30 cm) were collected and surfaced sterilized; bud meristems were dissected from the cuttings under a binocular microscope and transferred onto the induction medium in Petri dishes. The induction medium contained the inorganic and organic constituents of medium of Murashige & Skoog (1962), 5 μM thiamine-HCl, 80 μM myo-Inositol, 0.7% of Difco Bactoagar, 3% sucrose and 5 μM of 2,4-D. The pH was adjusted to 5.7 prior to autoclaving at 115 C for 15 min. After 24-48 days the callus induced was transferred onto 2,4-D free medium to permit embryo formation. Somatic embryogenesis capability was found to be genotype-dependent since the number of embryos obtained was very different in the five clones tested. In the clone Q23728, where this capability was high, clusters of fused and individual embryos at different stages of development were observed on 87% of the calluses, with a mean number of embryoids per explant of 7.4; 236 plantlets and 165 completely developed plants were obtained. So far, in sweet potato, no evidence has been reported that somatic embryogenesis, induced through an intermediary callus phase, leads to genetic variability in the regenerated plants. Studies are now in progress to evaluate whether the regeneration progress induced genetic variability.

CACAO SOMATIC EMBRYOGENESIS

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Cacao has been cultivated for more than 400 years and this crop still faces numerous problems with diseases, pests and the lack of high yielding clone materials. In vitro culture methods could play an important role to complement existing breeding efforts and to establish cacao plantations with superior germplasm.

Recent progress has been made on the development of methods for production of somatic embryos (SE) from non-sexual explants (petals and nucellus tissues). In addition, progress has been made to ensure that cacao SE complete germination and plantlet development phases leading to the recovery of adult plants. Large numbers of SE have been produced and resulting cacao plants have been raised to maturity. The regeneration rate has been 4.3% for petals (9,000 explants) and 2.0% for nucellus (29,000 explants). Embryogenic liquid cultures have also been established and the frequency of 8.8% SE/colony was recorded 4 weeks after plating. Extrapolating this initial data, it is expected to achieve a yield of 15,658 cacao embryos per liter per month.

This progress now offer the opportunity for fine-tuning micropropagation methods for elite cacao trees. Mature donor plants could be selected from segregating field collections and submitted to a cloning process. Other cacao improvement programs that would rely on cell and molecular genetic methods can also use these improved methods starting from selected donor plants.

IN VITRO PROPAGATION OF *AVERRHOA CARAMBOLA* - A TROPICAL FRUIT TREE

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The carambola, ***Averrhoa carambola***, belonging to the family Oxalidaceae is one of the common fruit trees which are native to humid tropices of the South East Asia. Vegetative propagation of the plant is desirable as the seedling trees are highly heterogenous. Proliferating cultures of both the axillary and adventitious shoots were established on MS and modified MS media from explants of in vitro raised seedlings with different concentrations of BA and NAA. Seedlings grown on media containing 4.4 µM BA were with bushy shoots but reduced hypocotyls and roots. Nodal segments from these seedlings were found to be the best for axillary shoot formation on agar gelled MS medium with ½ major salts and 4.4 µM BA. Hypocotyl explants from the seedlings raised on BA-free medium were proved to be the most suitable for regenerating adventitious shoots on the same medium with 4.4-2.2 µM BA and 0.5-2.5 µM NAA. After 10–12 weeks of sequential reculturing and subculturing 20-30 usable axillary/adventitious shoots could be produced from each 1 cm nodal/hypocotyl explant. The in vitro proliferated shoots were rooted on ½ strength MS medium having 0.5-2.5 µM NAA or IBA. Ninety per cent rooting success with 2-4 roots per cutting was achieved at 1.0 µM IBA on the same medium. NAA in the medium produced callus formation at the microcutting bases thus inhibiting emergence and normal growth of roots while IBA produced no callus and favoured normal growth and development of the roots.

CELL SUSPENSION CULTURES IN STRAWBERRY, GROWTH CHARACTERIZATION AND VARIABILITY

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Plantlets from callus culture of strawberry have been obtained for several varieties; however, it is still difficult to induce regeneration in cell suspension. Callus cultures were prepared by incubating anthers (adult plants), leaves, petioles and stems (in vitro grown plants), on basal solid medium by Gresshof and Doy (1974) with hormones at different dosages and combinations. The highest callus production was obtained with either 25 μM kinetin plus 10 μM NAA or 5 μM BA plus 10 μM NAA. Callus was then transferred to liquid culture for the establishment of cell suspensions. The growth and the variability of the cultures were evaluated through cell concentration measures (cell counting and packed cell volume) and viability was estimated by the fluorescein diacetate method. The initial cell concentration necessary for rapid growth and the growth curves of several cell lines of the same genotype were determined. Microscopic observations revealed that two cell lines differ in cell size, starch accumulation and viability. Isofocusing of cell extracts and medium, done at different points of the growth curves, did not reveal polymorphism in peroxidase isoenzymes of the cell but two isozymes were found in the medium which were slightly different in pI from those of the cells.

ACTIVATED CHARCOAL IMPROVES THE IN VITRO CULTURE OF VANILLA

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Vanilla (Vanilla planifolia Andrews) is an herbaceous perennial vine which produces the flavouring material and spice used for ice-cream. The plant is attacked by a root rot disease (Fusarium batatatis) which can completely destroy the plantations. Clonal propagation is normally done by cuttings, although attempts have been made recently by using tissue culture techniques. The objective of this work was to determine if activated charcoal could improve the in vitro culture of vanilla. Shoot apices of vines grown in the laboratory were cultured on Murashige and Skoog medium supplemented with 1 μM indolebutryic acid + 0.3 μM gibberellic acid + 2.2 μM benzyladenine with or without 5 g/liter activated charcoal (+/- AC). After several evaluations we found the best responses when AC was present in the medium. For example, % contamination was 0% + AC and 10% -AC, % survival was 96.6% + AC and 86.6% - AC, % rooting was 79.9% + AC and 36.6% - AC, root number was 0.89 + AC and 0.40 - AC, root length was 6.69 cm + AC and 1.01 cm - AC, and shoot length was 3.17 cm + AC and 3.07 cm - AC. Plantlets developed on medium + AC could be successfully transplanted and grown on soil mixture, before taking them for use in plantations and further evaluations.

THE EFFECT OF MOTHER PLANT PRETREATMENT AND EXPLANT CHOICE ON REGENERATION FROM IN VITRO PEAR LEAVES.

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Adventitious bud regeneration has been obtained at high rates from in vitro leaves of 3 pear cultivars (Conference, Comice and Passe Crassane), but with a low level of reproducibility. The effect of various pretreatments of the mother plants on leaf regeneration ability was tested. Horizontal position during subculturing produced numerous but shorter shoots, and induced a lower rate of regeneration from the leaves as compared with vertical position. Among 3 cytokinins tested (BA, kinetin, 2iP), BA at 2 µM, induced a higher rate of multiplication and shoot elongation of the mother plants, and a greater rate of regeneration from leaves. Reducing the interval between subcultures from 40 to 20 days before sectioning the explants significantly decreased the rate of regeneration. Leaves taken from the upper most part of shoots had a higher rate of regeneration and produced the highest number of buds per regenerating explant. The conditioning of the mother plants proved to be an important factor to increase leaf regeneration responses while reducing their variability.

SYNCHRONIZATION OF SOMATIC EMBRYOGENESIS IN TEA (*Thea sinensis* L.)

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Synchronization of the somatic embryogenesis in tea is important for developing a automation system, and other genetic studies. Recently, we have reported potential of peripheral cells of the cotyledonary explant to form somatic embryos in tea (Zakia et al 1991). Embryogenic tissues in liquid culture medium maintained at 160 rpm, kept in dark with increased sucrose concentration (8%) resulted in the synchronized development of embryos. After 28 days from each batch of culture, the percentage of torpedo shaped embryos recovered was about 90%. PAGE analysis of cultured somatic embryos, at the different stages of development, exhibited specific patterns of bands for buffer soluble proteins, and isozymes i.e. peroxidase, esterase, and dehydrogenase. Qualitative analysis of the spectrum of buffer soluble proteins and isozymes could be used as a biochemical marker for evaluating the developmental stages of somatic embryos in an embryogenic culture.

Ref: Zakia, B., Rajarathnam, S. and Mohanty, B.D. 1991. J. Hort. Sci. 66 (4) 465-470.

MICROPROPAGATION AND IN VITRO PRODUCTION OF NOVELTIES OF COMMERCIALLY ATTRACTIVE ENDANGERED CACTI.

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The Cactaceae is a family with valuable ornamentals which contains medicinal, industrial and edible species. Many of the members of this unique group are on the edge of extinction due to habitat destruction and illegal overcollection. Plant biotechnology methodologies can help stop the disappearance of these endangered species. The aims of this work were to develop biotechnology tools that increase the availability of endangered cacti in the international market as well as enhance the value of the in vitro derived plants making them more commercially attractive. Several explants from selected species were cultured on MS media supplemented with growth regulators in order to be mass-propagated. Rooting was a problem for most of the species but in the case of Mammillaria san-angelensis and Echinocereus, well developed roots were produced and the plants adapted to greenhouse conditions. Axillary shoot proliferation rendered clonal plants, but epigenetic changes were also obtained in some cases. These changes are commercially valuable and could be offered as "rarities" of rare plants to specialized collectors: unusual bouquets (3-25 shoots rooted together of M. san-angelensis and Echinocereus) and cristates forms (Escobaria) were obtained. Also, increases in proliferation rates of Aztekium (reputed to be the slowest growing cactus) have been achieved. Financial studies have been made showing that micropropagation is highly profitable and requires little capital.

ROLE OF EXPLANT TYPE AND ACTIVATED CHARCOAL IN PROPAGATION OF DATE PALM BY TISSUE CULTURE

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Aseptic explants from various sources of tissue obtained from clonal date palm offshoots of cv. "Sefri" were cultured on MS inorganic salts, supplemented with 87.6 mM sucrose, 1.2 μM thiamine-HCl, 555.1 μM 1-inositol, 44.4 μM BA, 16.1 μM NAA, 38.9 μM glycine, 3 g/l activated charcoal and 7 g/l prewashed agar. The morphogenic response of the various sources of explants was found to depend on the source of the explant, the illumination of culture during incubation and explant wounding prior to culturing.

Shoot tips and lateral buds, of cv. "Sefri" were established on MS basal medium and 87.6 mM sucrose, 1.2 μM thiamine-HCl, 555.1 μM 1-inositol, 16.1 μM NAA, 22.2 μM BA and 38.9 μM glycine. Different concentrations of activated charcoal (0.0, 0.5, 1.5 and 3.0 g/liter) in liquid and agar solidified media were tested. Concentration of 1.5 g/liter charcoal was found to be superior for vegetative growth of buds. The growth of the buds in liquid media was much better than in agar-solidified media in all concentrations of charcoal tested.

INCREASING BUD REGENERATION IN AN ORNAMENTAL CAMELLIA

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The effect of calcium ionophore A23187 dissolved in DMSO (Dimethyl Sulfoxide, 0.2% total volume), on the induction of regenerant buds in an ornamental camellia (*Camellia x williamsii* cv Debbie) was investigated.

Leaf and stem sections derived from *in vitro* or *in vivo* plants were cultured on MS medium supplemented with 18 µM BA, 10 µM IBA and A23187 (0, 10, 20 µM), only in the regeneration medium or in both callus inducing and regeneration media.

The number of regenerant calluses was enhanced both by A23187 and DMSO alone compared to the control. The number of buds per regenerant calluses was highly increased when A23187 was used both in callus inducing and regenerant medium.

PROPAGATION OF FLOOD TOLERANT JACKFRUIT(*ARTOCARPUS HETEROPHYLLUS*) BY IN VITRO CULTURE

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In vitro culture method for the propagation of flood tolerant jackfruit (*Artocarpus heterophyllus*) has been acheived. Jackfruit plant is highly sensitive to water-logging. The plant is usually propagated by seeds. Budding, grafting and cutting are unsuccessful. As the plant is predominantly outbreeding, the characters of the plant differ widely in different individuals. We have earmarked some flood-tolerant individuals and in vitro culture method has been employed for its clonal propagation, using axillary shoot buds as explants. Results showed that addition of BAP at 8.88 μM and NAA at 2.65 μM to MS medium induced maximum number of shoot buds. For inducing axial growth in regenerated shoot buds, the hormone concentration of the medium was lowered and coconutmilk at 10% (v/v) was added. Rooting was best in ½ strength MS medium containing 5.3 μM NAA and 4.9 μM IBA. The percentage of both rooting of shoots and survival of the rooted shoots was 75-80%. Continuous trials using explants from the elite trees throughout the year showed that the period between June-August was the best season for the explant source for rapid and increased multiplication of axillary buds.

THE ACCLIMATIZATION OF IN VITRO CULTURED PLANTLETS IN THE BREEDING OF SEEDLESS TABLE GRAPES (VITIS VINIFERA) IN SOUTH AFRICA.

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An increasing demand for seedless table grape varieties has focussed the attention on the breeding of new and improved cultivars. In seedless cultivars the embryo aborts several weeks after flowering. Conventional breeding between seedless (male) and seeded (female) parents resulted in only 10 - 15 % seedlessness. The embryo rescue technique, however, enables the breeder to cross seedless parents resulting in a higher percentage seedlessness than the conventional method. Ovules were removed from the developing berries 4 weeks after flowering and cultivated on Nitsch and Nitsch medium. After 12 weeks the embryos were removed from the ovules and allowed to germinate. The acclimatization of these in vitro cultured plantlets, however, posed a problem as high mortality rates were observed. In order to establish a standardized method of acclimatization, a model system was developed investigating a) the conventional method of acclimatization, b) the adapted acclimatization method of Goussard and Wiid (1989), c) the effect of different soil mixtures, d) the effect of different plastic enclosure periods and e) the effect of Vapor Gard (a film forming polymer of x-pinene acting as an anti-transpirant). The conventional method was preferred to the adapted Goussard/Wiid method as the latter proved to be time consuming and labour intensive. Potting mixtures of speedling mix and vermiculite or perlite resulted in 90 - 100 % survival of plantlets. A plastic enclosure period of 9 - 12 days proved to be optimum. The use of Vapor Gard, however, resulted in high survival rates and the plantlets were healthy and normal. The data indicate that Vapor Gard can be used successfully to ensure a high survival rate of plantlets and could eliminate time consuming and labour intensive acclimatization procedures.

SOMATIC EMBRYOGENESIS FROM LEAVES OF LISIANTHUS RUSSELLIANUS HOOK

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After 45 days of culture on MS basal medium plus 2,4-D (4.5-18 μM), friable callus was obtained from leaves of in vitro grown shoots of Lisianthus russellianus Hook clones. The callus was utilized to establish suspension cultures with high viability (FDA test) on media containing the same concentrations of 2,4-D. Suspensions were filtered through a 200 μm sieve to select the smallest clusters and single cells; globular structures developed in 30 days. These structures, collected on a 500 μm sieve were able to produce torpedo shaped somatic embryos when transferred to 2,4-D-free medium. Among the concentrations tested of 2,4-D, 9 μM was the best, either for callus formation or somatic embryo production. Continuous dark improved callus friability and reduced the aberrant forms of the embryos in comparison to 16/8 hours light/dark cycles. Germination of somatic embryos was the critical step of the protocol; only 5% were able to synchronize their shoot and root development; more commonly (45%), the root apex developed first followed by the regeneration of the shoot. These plantlets were easily acclimatized in the greenhouse. Experiments are in progress to increase embryo yield and to synchronize germination.

CYTOLOGICAL AND MOLECULAR STUDIES ON THE PROCESSES UNDERLYING COMMITMENT IN PLANT SOMATIC EMBRYOGENESIS

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Microdensitometric and cytological data have suggested that carrot somatic embryogenesis could be correlated with the occurrence of meiotic-like segregational division. More over, partial DNA loss per nucleus during the acquisition of embryogenic competence could be inferred. Further histological observations on hypocotyl explants of carrot and other species, cultured in presence of 2,4-dichlorophenoxyacetic acid (2,4D), have also shown the differentiation, in concomitance with the segregating events, of structures resembling reproductive organs such as ovaries and anthers.
Molecular research is in progress to confirm the partial DNA loss and to specify the nature of the sequences involved. Special attention will be focused on highly and moderately repetitive sequences, owing to their known plasticity and variability in response to different kinds of stressing conditions including cell culture, Moreover several different probes can help to elucidate the characteristics of these phenomena related to commitment.

EMBRYOGENESIS IN DIFFERENT EXPLANTS OF CERTAIN INDUCED MUTANTS OF*HELIANTHUS ANNUS*.L.

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Certain mutants from two varieties of sunflower, var. S.S.56 and CO-2 were assessed for efficiency in embryogenesis with different media and hormones. The different explants tested were cotyledonary leaves, root tips, immature embryos, mature embryos, transition zones, shoots, and leaf bases. The efficiency of these explants varied significantly. It was maximum for immature embryos and minimum for the root tips in both varieties. Of the different media and hormones tested, pronounced embryogenesis was observed in MS-modified media supplemented with NAA + kinetin and NAA + GA3 + BA.

ORGANOGENESIS IN SINGLE LEAF CULTURES OF *NARCISSUS*

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Organogenesis of *Narcissus* can be achieved via shoot induction from the basal plate region of single leaf explants. This is normally followed by the in vitro formation of bulbils on the resultant shoot clumps, to aid transfer to *in vivo* conditions. Direct bulbil organogenesis on single leaf explants would enable development of a system for large scale clonal multiplication of *Narcissus* in vitro.

The effects of sucrose (3, 6 and 9%), NAA (0 and 0.5 uM) and BA (0 and 4.4 uM) on organogenesis were examined using single leaf explants excised from shoot clump cultures of *Narcissus* cultivars St. Keverne and Hawera. Direct bulbil production was stimulated by the combination of NAA with either 6 or 9% sucrose. No bulbils were produced at the lowest sucrose concentration. Omission of NAA severely reduced the number of bulbils formed. BA was inhibitory to bulbil formation throughout.

Shoot, root and bulbil formation could be effectively manipulated by modifying the amounts of the sucrose and hormones in the culture medium.

SUCCESSIVE PHASES DURING ROOTING OF MICROCUTTINGS OF *MALUS*

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Regeneration of roots can be divided into three phases, viz., dedifferentiation, differentiation and outgrowth. It is likely that cytokinin added to the rooting medium interferes with the second phase. We determined during which period BA had the maximum inhibitory effect on rooting of microcuttings of *Malus* 'Jork'. When BA was added to the rooting medium from 0 to 24 h, 72 to 96 h or 96 to 120 h after transfer of the microcuttings to rooting medium, the effect was only small. When BA was added from 24 to 48 h or from 48 to 72 h, rooting was almost completely inhibited. Thus, the BA-sensitive phase was between 24 and 72 h. The concentration of BA required for inhibition was 2 μM or higher. We are examining on the microscopic level how far the rooting process has progressed by 48 and 72 h and how regeneration is disturbed by BA. Data from the same apple cultivar - albeit rooted under somewhat different conditions - indicate that the first cell divisions occur circa 48 h after transfer to the rooting medium. When pulses of IBA were given, best rooting was obtained with pulses from 24 to 48 h or from 48 to 72 h. However, the promotive effect of IBA was not as clear as the inhibitory effect of BA, and was only observed when a high concentration of IBA (3 μM) was supplied. We are currently examining the effects of ethylene, $AgNO_3$ and 2,4-D given as pulses.

COFFEE SOMATIC EMBRYOGENESIS IN LIQUID CULTURES

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Despite coffee's great commercial importance, a commercial micropropagation method is not available for this crop. DNAP (USA) and SPIC (India) have embarked on a program to develop a process for large-scale micropropagation of coffee.

Friable embryogenic tissue (FET) isolated from coffee leaf explants and maintained on solid medium for 3 months was inoculated in liquid medium for proliferation and embryo differentiation. Cell mass increase was accomplished in a medium containing 1 µM 2,4-D and 0.5 µM kinetin. Liquid medium was replaced after the first 4 weeks in culture and globular embryos were observed following an additional 4 weeks. At this time, the liquid medium was changed to the same basic medium supplemented with 2.6 mg/l ABA and replaced with fresh medium 4 weeks later.

In 12 weeks, FET cultures initiated at a low density increased 20 fold in packed cell volume. After this period, torpedo stage embryos were observed at a low frequency. Cultures kept without any medium exchange for additional 9 weeks differentiated entirely into embryos. Somatic embryos at different stages of differentiation were subcultured into liquid maturation medium for 4 weeks with a two-week subculture schedule. Mature embryos were plated on solid germination medium as individual embryos or in clusters. Eight weeks later, individual embryos increased 2-3 fold in size and presented normal morphology. In contrast, embryos plated in clusters were characterized by a delayed growth. Preliminary data revealed a yield of 2,500 embryos from an original inoculum of 0.2 g f.w. FET cells. Extrapolating this initial result, to acheve a micropropagation target of 1 million coffee embryos would require the use of 80 g f.w. FET cells cultured in four 5-liter bioreactor vessels.

ORGANOGENESIS AND REGENERATION OF SOME ANDEAN FRUIT SPECIES.

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Lesser-known Andean species are of potential economic importance, due to their fruit quality, flavor and vitamin content. The regeneration of elite clones is required to establish standard and consistently productive plantations. In vitro experiments on different cultivars and ecotypes of three promising species were conducted in our laboratory. Results have shown, for Annona cherimola (cherimoya), primarily multiple shoots were formed directly from hypocotyls, which showed strong polarity. Callus formation also occurred in different explants, in spite of the high oxidation rate shown by this species. Browning has been strongly reduced by antioxidant supplements. Solanum muricatum (pepino) shows great organogenic potential; leaves cultured with different hormone supplements regenerate roots and shoots, leading to plantlets in 33% of explants. No subculturing is required. Preliminary results show that Cypomandra betacea (tamarillo) axillary bud explants also show high regeneration potential, forming shoots and roots. Recovery of plantlets of these species under in vitro conditions is continuing in our laboratory. Transformation research has also been started.

Acknowledgements. Partial support for this presentation was provided by AID Grant 513-5541-G-SS-9067-00.

Session V:

Manipulation of Protoplasts and the Haploid Genome

WOODY PLANT PROTOPLAST TECHNOLOGY REVISITED

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After nearly three decades of basic protoplast research, it is now clear that the potential for economic gain through protoplast manipulation will finally be fulfilled and the technology progressively accepted by plant breeders and seed companies. Progress in the application of protoplast technology to the improvement of cultivated crops is still somewhat restricted by a lack of plant regeneration in some species despite notable advances and breakthroughs, in recent times, with respect to the cereals and woody species. Interestingly, though, the small fruit crops (notably the grapevine), many vegetable crops and species of floral value, the monocotyledonous species and most woody plant genotypes are in general difficult to deal with, and the establishment of reproducible protoplast-to-plant systems is still largely achieved by empirical means. There is a compelling case to increase our knowledge as to the role that complex media play in triggering sustained division in protoplast-derived cells, and also of the relationships between these factors and the genetic basis of plant regeneration. This paper will summarize and critically examine recent advances in protoplast technology within the context of breeding horticultural crops and, mainly, as applied to deciduous temperate fruit tree species.

CITRUS SCION AND ROOTSTOCK IMPROVEMENT VIA SOMATIC HYBRIDIZATION

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The technique of protoplast fusion has been used to produce numerous Citrus somatic hybrids that have potential in citrus scion and rootstock improvement, including interspecific hybrids, and intergeneric hybrids between both sexually compatible and incompatible parents. The general method used to generate Citrus somatic hybrids is polyethylene glycol (PEG) induced chemical fusion of competent Citrus protoplasts isolated from nucellar-derived friable embryogenic callus or suspension cultures of one parent with protoplasts of a second parent isolated from seedling leaves. Verification of somatic hybridity is provided by chromosome number determination in combination with electrophoretic analyses of either isozymes or DNA banding patterns. Strategies for the application of somatic hybridization to citrus rootstock improvement include the production of allotetraploid hybrids that combine complementary rootstock genotypes, and wide hybridization of Citrus with species from related genera that possess traits of interest. The major strategy for scion improvement is the production of interspecific somatic hybrids that combine complementary parents for use in interploid crosses to generate seedless triploid zygotic progeny. This review will describe the somatic Citrus hybrids produced to date and discuss their potential value to citrus scion and rootstock improvement. Preliminary observations on somatic hybrid fertility and field performance will also be presented.

POTATO HAPLOIDS IN GENETICS AND BREEDING

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Haploids from polyploids such as potato (Solanum tuberosum) that have four sets of similar chromosomes (tetrasomic polyploids) offer unusual opportunities for genetic and breeding research. This is due to the fact that the genetic and cytogenetic behavior of these haploids is that of normal diploids. Haploids are particularly valuable in the genus Solanum which includes diploid species closely related to the economically important tetrasomic polyploid. Thus, for genetic and breeding research haploids (2n=2x=24) of the common potato (2n=4x=48) have been used more extensively than haploids of any other crop plant. They have been used to help solve problems related to the cytogenetics, genetics, evolution germplasm utilization and breeding of the Solanums. Specifically, in cytogenetics they have been employed in research on the nature of ploidy, basic chromosome number, aneuploids and monoploids; in genetics - disomic ratios, gametic samples, inter- and intralocus interactions and molecular mapping; in evolution - origin of the cultivated potato, genetic relations of wild and cultivated potatoes and chromosome differentiation among taxons; in germplasm utilization - evaluation of germplasm, enhancement of germplasm, increased allelic diversity and selection at the 2x level; in breeding - germplasm transfer from wild to cultivated species, comparing asexual with sexual polyploidization and as a method of incorporating a higher percentage of adapted germplasm.

PLOIDY CHANGES IN "MITCHELL" PETUNIA.

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The haploid "Mitchell" Petunia was created by Mitchell, Hanson, Skvirsky and Ausubel (A. Pflanzenphysiol. Bdt. 1005, 1980:131-146) from anther culture. Haploid organisms are useful in genetic studies because they allow the expression of recessive alleles. In an effort to isolate a cell cycle mutant of Petunia for biochemical studies, protoplasts were isolated from the haploid Mitchell Petunia plant. Initially the haploid Mitchell Petunia was propagated in the greenhouse and in culture and the resulting plants checked for ploidy using three methods: chromosome counts of root tips, number of chloroplasts per guard cell pair, and microfluorimetry. The majority 80% of haploid plants propagated through tissue culture were chimeric rather than either purely diploid or haploid. Cultured cells that were initiated from leaves of diploid plants also changed their ploidy and became predominantly tetraploid. Both tetraploid and diploid mesophyll protoplasts grew in culture at the same plating efficiencies.

SOMATIC HYBRIDS AND CYBRIDS BETWEEN SENECIO FUCHSII GMEL. AND SENECIO JACOBAEA L.

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Somatic hybridization between Senecio fuchsii and S. jacobaea was investigated. Regeneration from protoplasts had been achieved with both species (Binding et al., 1981 and 1991). In order to establish visual markers, albino mutants of S. jacobaea (one plastid and one nucleus mutant) were selected after treatment of protoplasts with nitromethylurea. Fusion was obtained via the agarose sandwich lens technique with high Ca++ and high pH (Binding et al., 1988). Several regenerant lines were selected by pigmentation and morphological characteristics. The constitution of five hybrid lines and two cybrid lines were confirmed by chromosome numbers, patterns of isozymes (peroxidase, esterase, malate dehydrogenase, glutamicoxalacetic transaminase), and RFLPs. Restriction fragment patterns of total DNA were explored by specific probes of nuclear DNA and chloroplast DNA. Plastids had segregated in the shoots investigated. The two cybrids each possessed 2n=2x=40 chromosomes. One of them contained the S. jacobaea nuclear genome and S. fuchsii plastome, the other one contained S. fuchsii nuclear genome and S. jacobaea plastome. Analyses of RFLPs with mtDNA probes are in progress. The development of plants in the greenhouse is being followed.

GENETIC IMPROVEMENT OF EUROPEAN PEAR ROOTSTOCKS

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As part of the programme for the rapid genetic improvement of Quince A [*Cydonia oblonga* Mill.] as rootstock for pear, an efficient protoplast-to-tree system is being developed. Stem callus produced from axenic shoots on multiplication medium could be induced to undergo sustained caulogenesis on a medium containing GA_3 in addition to IBA and BA. Elongated shoots were rooted *in vitro* and plantlets established in the glasshouse. Suspension cultures were initiated from white friable stem callus produced on UMB medium [MS salts,87.6mM sucrose, 2.27µM 2,4-D 1.16µM kinetin, pH5.8] at 25°C with a 16h photoperiod [2.5 m^{-2} s^{-1}]. The removal of ammonium ions from the MS salt formulation [UMB-NH_4] produced fast growing suspension cultures from which high yields of viable protoplasts can now be routinely isolated. Culture of these protoplasts in ammonium-free media containing antioxidants has produced IPE values of maximum 10% and cultural conditions promoting sustained division and microcallus production are being investigated.

ISOLATED MICROSPORE CULTURE OF *COFFEA ARABICA* L.

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The most cultivated coffee (*Coffea arabica* L., 2n=4x=44) is an autogamous perennial. The traditionnal breeding schemes are very slow as they are based on pollinations followed by 5 to 7 selfings. The utilization of doubled-haploïd plants should lead to pure lines more directly. Moreover, induced mutations or somaclonal variations of eventual interest at the haploïd state would be fixed as homozygous by the doubling process.
Some studies on anther culture in coffee have led to the conclusion that the diploïd tissues react very quickly to the tissue culture process by forming diploïd somatic embryos, thus preventing the microspore reaction. For that purpose, we investigated the possibilities of microspore isolation and culture. Microspores were isolated using a commercial blender and a 48 µm sieve. Important phenolic oxidations were found but appropriate anti-oxidant use overcame this problem. We demonstrate the feasability of the method. To our knowledge, it is the first time such a technique is reported in coffee.

TOWARDS THE SOMATIC HYBRIDIZATION OF SHRUBBY AND CLIMBING HONEYSUCKLES

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The genus *Lonicera* includes several shrubby (with small, unperfumed flowers) and climbing (with big, scented flowers) species which are among the most widely cultivated ornamentals in Europe. It would be desirable to create shrubby hybrids with big perfumed flowers. This, though, has proved elusive to date, on account of incompatibility problems that preclude natural gene flow between species. Protoplast fusion, with the further recovery of novel somatic hybrids appeared as a feasible alternative to overcome the existing barriers to sexual crossing. However, the development of protoplast-to-plant systems for the prospective fusion partners is a prerequisite for such goal. Against this background, large numbers (> 10^6/g f.w.) of highly viable (> 80 %) protoplasts were isolated from in vitro leaves, callus tissues and cell suspensions of the shrubby species *L. fragrantissima* and the climbing genotypes *L. periclymenum* cv. serotina, and *L. x brownii* cv. "Dropmore Scarlett". The requirements for protoplast isolation for each genotype and source tissue were established. The cultured protoplasts divided to produce small cell colonies and then microcalluses. The percentage plating efficiency, at various developmental stages, was assessed for protoplasts of all genotypes and sources. Experiments aimed at the further growth and subsequent organogenesis from such, protoplast-derived honeysuckle tissues are now underway.

Session VI:

Strategies for the Use of Somaclonal Variation

SOMACLONAL VARIATION: HAS IT PROVED USEFUL FOR PLANT IMPROVEMENT?
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Somaclonal variation involves all forms of variation encountered in tissue culture. Somaclonal variation has proved to be a serious problem for investigators and propagators who require extreme uniformity. However, the natural variability associated with tissue culture represents a pool upon which selection pressure can be imposed to isolate unique forms of a clone. The origins of somaclonal variation have been studied extensively, but remain largely theoretical or unknown. Some types of variability are not stable (epigenetic); others have been stable through repeated generations of asexual propagation; and many somaclonal variants have proved to be genetically based and inherited in a Mendelian fashion. Some somaclones have been formally introduced as improved types of the original parental clone. Some of these unique plants will be discussed.

The amount of variation that can be expected in vitro will vary with the clone, age of the culture, use of mutagenic agents, and use of selection pressure applied to single cell clones for stress conditions such as salt level, herbicides, microorganisms or their byproducts, and specific metabolites. Somaclonal variation has been reported by so many researchers working with so many different crops, that it is reasonable to expect somaclonal variation in all tissue culture experiments. The extent, causes and proven usefulness of somaclonal variation will be discussed during the presentation.

DISEASE RESISTANCE, CELL CULTURE AND SOMATIC RECOMBINATION

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As breeding of a particular crop becomes more intensive or has a longer history, it is usual to rely more heavily on variability beyond the species; so-called *alien* germplasm. Attendant with this shift to alien genes come the constraints of recombination. It is rarely useful to make wide hybrids unless it is also possible to obtain genetic recombination between the crop and alien chromosomes. Restrictions of meiotic recombination frequently preclude the deployment of useful alien genes from wide hybrids into new cultivars. Somatic recombination is an alternative means to obtain transfer of useful genes from an alien genome to the crop chromosomes. Cell culture of alien chromosome addition lines has proved a useful means to induce somatic recombination.

Barley yellow dwarf virus (BYDV) resistance was located on a particular *Thinopyrum (Agropyron) intermedium* chromosome. Cereal cyst nematode (*Heterodera avenae*) resistance was located on a rye (*Secale cereale*) chromosome. Monosomic addition lines to wheat were constructed carrying the resistance chromosome and the normal complement of wheat chromosomes. Immature embryos or inflorescences were cultured as callus and plants regenerated and selfed. Progeny families were screened for disease resistance segregation patterns. Families have been identified which have no alien chromosome but carry the resistance on a recombinant wheat chromosome.

Protoplast fusion is an alternative starting point for somatic recombination. We have attempted to establish this with fusions between *Medicago sativa* (alfalfa) and *Onobrychis viciifolia* (sainfoin). The *donor* sainfoin protoplasts were first gamma irradiated to disturb the nuclear organisation and limit the input of *alien* chromatin. Total genomic DNA probes are indicating the presence of small amounts of sainfoin chromatin in the alfalfa-like regenerated plants.

SOMACLONAL VARIATION IN BREEDING FOR DISEASE RESISTANCE IN TOMATO

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Disease resistant plants have been obtained by the screening of tissue culture derived plants, or their progenies, at the whole plant level. The efficiency of the selection procedure can be increased when it is combined with *in vitro* selection. The perspectives of these strategies were investigated in order to obtain tomato plants with resistance to bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis*. The extracellular polysaccharide produced by the pathogen was isolated and characterized. Although it showed phytotoxic activity in a bioassay, it was not suitable for use as a selective agent. Subsequently somaclones were regenerated from leaf, cotyledon and hypocotyl explants of the susceptible tomato cv. Moneymaker. Various phenotypic alterations were observed among the regenerated plants (R1), but were not transmitted to the progenies. Several monogenic, recessive mutations were recovered in the R2 populations, four of which were shown to be allelic to known recessive single gene mutants. Also, aberrations of ploidy level, mainly tetraploidy, occurred. Explant source did not influence mutation frequency or mutation spectrum.

Since genetic variation was observed, progenies of the somaclones were tested for resistance to bacterial canker. A fast screening method in the greenhouse, with a criterion for the selection of single, putatively resistant plants based on the severity of wilting symptoms, was used. The evaluation of progenies of 279 somaclones for resistance showed that some variation for severity of wilting was present. However, somaclones with a major increase in resistance, and thus valuable for plant breeding, were not found. The results suggest that the potential of somaclonal variation as a source of resistance to bacterial canker is limited.

FACTORS INFLUENCING THE OCCURRENCE OF SOMACLONAL VARIATION IN MICROPROPAGATED BANANAS

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Initial explants were cultured on 16 different media and, during a period of 420 days, plantlets were subcultured every 6 weeks. Plantlets developed were divided into two classes containing: (a) single, fully developed large plantlets (L) which form a bulbous-like corm: and (b) plantlets whose growth and corm formation is suppressed and resulted in formation of a few to many basal small plantlets (A) without distinct basal corm (suspected to be adventitious). The bulbous-like growth of the L plantlets was split longitudinally when they reached a size of 3 leaves. Each half was subcultured for further multiplication. During the period A plantlets were separated from the L plantlets and transferred to the field. Some of the L plantlets were also transferred to the field at different subculture and all of them at its termination. The following details were kept for each of 4136 field evaluated plants: kind of medium and plantlet (L or A), generation and initial explant number. The main conclusions were: (a) the rate of variants obtained was not affected by medium composition or by the rate of multiplication; (b) the rate of variants was lowest in L plants and much higher in A plants; (c) the length of time in culture was not found as a mutation inducing factor since some variants were found in plants derived from the initial explants; and (d) the initial explant was found to be the main factor which determined the occurrence of somaclonal variants. Stable and non-stable families were obtained from various explants.

SOMACLONAL VARIATION IN ORNAMENTAL PLANTS

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Our aim of this investigation was to evaluate somaclonal variation in Begonia x elatior and Saintpaulia plants for flower color, flowering time, flower morphology, number of flowers per plant. Sterilized leaf disks of Saintpaulia were cultured for direct shoot induction on MS medium containing 0.5 μM BA and were rooted on MS medium amended with 0.6 μM NAA. Over 1000 regenerated plantlets were evaluated in the greenhouse. No variation in flower color and size was seen. However, there were differences in flower morphology, number of petals/flower, flowering time and number of flowers/plant. Plants flowered as early as 78 days and as late as 109 days. About 47% regenerated plants had no flowers and 0.4% plants had an average of 31 flowers/plant. Flower corolla was tubular in 3-4% plants and abcissed by a gentle touch. Non-flowering plants had hard, thick, and sometimes variegated leaves and their growth was slow. From Begonia leaf disks, callus was initiated on MS medium supplemented with 0.6 μM NAA, 0.9 μM 2,4-D, and 0.5 μM BA. After 4-5 weeks, Begonia callus was differentiated into shoots on B5 medium having 0.5 μM zeatin and 5.0 μM kinetin. Regenerated shoots were rooted on MS medium containing 0.6 μM NAA. A total of 336 regenerants were evaluated in the greenhouse. No variation in flower color was seen. However, the differences were recorded in 2% regenerants for flower numbers (53 flowers per plant) as compared to the control (20 flowers/plant). Subsequently, selected plants of both flowering plants were micropropagated and resulted significantly stable lines. Our results indicate that flower color charcater of both Begonia and Saintpaulia seem to be stable under tissue culture conditions.

GENERATING TETRAPLOID MELONS FROM TISSUE CULTURE

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Tetraploid regenerants spontaneously occurred when melon (*Cucumis melo* L.) cotyledons were cultured on 10 μM BA. These polyploids were easily screened by pollen morphometry from precocious male flowers formed at the first few nodes. Less than 50% of single bud derived culture lines were mixoploid. Screening primary regenerants served as a good indicator of the ploidy of regenerants to come after 9 and 18 months of subculture. Thus, the origin of tetraploidy is prior to the subculture procedures. During this time, mixoploid cultures lost tetraploid members and shifted toward diploidy. Nuclear DNA content of original explant tissue was 1.7 picograms (2C) with no indications of populations at 4C. The absence of cell divisions and tetraploid cells in the explant tissue points to the early *in vitro* divisions as the source of tetraploids. The regenerant plants were non-chimeral with respect to ploidy and many were quite fertile. Triploid hybrids have been synthesized and these were sterile. The occurrence of tetraploidy as a function of developmental stage and region of explanted cotyledons will be discussed.

THE RESPONSE OF PEACH REGENERANTS, CULTIVARS, AND SEEDLINGS TO ROOT-KNOT NEMATODE

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Somaclonal variation has been reported in a wide number of plant species. A range of phenotypic, and genetic changes have been described including increased disease and pest resistance. The present study was designed to determine whether or not such variations were present in regenerated peach plants (Prunus persica [L.] Batsch). Several regenerants (156-1, 156-7, 156-11 and 156-12), obtained from immature embryo #156 from peach cultivar Sunhigh, were tested for root-knot nematode (Meloidogyne incognita) resistance in vitro. Nematode development and reproduction differed on these regenerants. Significantly higher numbers of nematodes developed on 156-7 compared with 156-12, 156-11 and 156-1. Lowest nematode development was observed on 156-1 and 156-11. M. incognita development on these regenerants was compared with in vitro propagated plantlets of susceptible cultivar Sunhigh, moderately resistant cultivar Redhaven and resistant cultivar Nemaguard. Significantly higher numbers of nematodes developed on cultivar Sunhigh and regenerant 156-7 compared with cultivar Redhaven and regenerants 156-1, 156-11 and 156-12. No nematode development was observed on Nemaguard. In greenhouse tests, although some nematode development was observed on 156-1, 156-12 and 156-7, nematode reproduction was absent or not observed. This research demonstrates the feasibility of screening for somaclonal variants of peach with increased levels of tolerance to M. incognita.

ISOENZYMATIC ANALYSIS OF SOMACLONAL VARIATION AMONG REGENERANTS FROM APPLE ROOTSTOCKS LEAF TISSUE

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Isoenzymatic analysis was used to evaluate genetic constitution of 5 apple clonal rootstocks (M.9, M.26, MM.106, MM.111 and Mark). Analyses were performed using leaf tissue collected from plants actively growing *in vivo* and *in vitro*. Isoenzymatic polymorphism was evaluated also among several *in vivo* growing regenerants from leaf callus of M.26 and MM.106. Eight isoenzymatic groups were considered for each clone: PGN, PGI, EST, APH, GDH, ENP, LAP, NDH and GOT.

Wide polymorphic variability was observed with APH and PGI groups and a good discrimination among the five rootstocks was possible. Some patterns seem to be influenced by the origin of the material: *in vivo* or *in vitro*.

The regenerants zymogram made possible to characterize differences among them and with the original clone.Considering the different field resistance to *Phytophthora cactorum* of the tested rootstocks, the difference observed in some patterns could be associated with the resistance to the disease.

Further analyses will be carried out to confirm this correlation and to verify if the observed polymorphism in the regenerants could be associated with an increased resistance to the fungus.

HERITABLE TISSUE CULTURE INDUCED VARIATION IN *ZINNIA MARYLANDICA*

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Zinnia marylandica adventitious shoots were regenerated from cotyledon tissue on Murashige-Skoog (MS) media containing 0.2 or 22.2 µM thidiazuron (TDZ) and grown through flowering to evaluate for variation. Fisher's Test for Equal Variance showed 0.2 µM TDZ-derived plants had more variation than seed-derived control plants for seven of nine parameters observed. Plants derived from 22.2 µM TDZ had more variation than control plants for five of nine parameters, and more than 0.2 µM TDZ-derived plants for two of nine parameters. Twelve of 149 0.2 µM TDZ-derived plants and three of 23 22.2 µM TDZ-derived plants had variant characters. Variants were self-pollinated and progeny observed for variation. Aberrant characteristics including plant height, fertility, and flower color and morphology were sexually transmitted in varying degrees, indicating genetic change had occurred. Aberrant characteristics not observed in regenerated plants arose in progeny. It is concluded that tissue culture regeneration of *Z. marylandica* plants by adventitious shoot formation on 0.2 µM TDZ-containing MS media successfully introduced heritable genetic variation.

RESISTANCE TO LEAF BLIGHT DISEASE IN SOMACLONES OF CARROT

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Leaf blight of carrot caused by the fungus *Alternaria dauci*, can be controlled by the use of fungicides. Currently no variety shows any significant level of resistance, but growers would prefer to plant more resistant cultivars and therefore overcome the need for fungicide treatment. One approach to increasing the level of variation, and hence resistance, is to pass the parent tissue through a tissue culture stage to induce somaclonal variation. Accordingly, callus cultures were initiated from stem sections of surface sterile seedlings of a main (U.K.) crop variety of carrot, Nantes Fancy, on an MS medium, 2% sucrose, 1.0 mg l^{-1} 2,4-D and 0.2 mg l^{-1} kinetin. Callus was maintained for two subcultures on this medium then transferred to the same medium with 2,4-D omitted to induce embryogenesis. Plantlets were separated and maintained singly until large enough to be transferred to sterile soil in the glasshouse. A total of 500 regenerant plants were grown to maturity. Shoot morphology (leaf shape, leaf number, shoot number, shoot height, flowering) and cytology (chromosome number) and response to infection (chlorophyll loss from infected excised leaves) was assessed. Regenerant plants showed wide variation in morphology and response to infection. The plants were vernalised for a 10 week period at 5-6^{o}C then allowed to flower and set seed. The progeny are currently being examined for variation in morphology, cytology, chlorophyll loss, and polyphenol accumulation in the leaves following infection.

SOMACLONAL VARIANTS OF GRAPEVINE OBTAINED BY SOMATIC EMBRYOGENESIS.

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Somaclonal variants of grape (*Vitis sp.*) were established by somatic embryogenesis from cv. Podarok Magaracha. An embryogenic callus was induced from leaves cultured on MS medium supplemented with 2,4-D (2.3-9.0μM) and BA (0.9-8.9μM). Somatic embryogenesis was induced after transferring callus to MS medium in the presence of NAA (5.4-10.8μM) and BA (2.2-8.9μM). Embryoids cultured on medium without growth regulators for two weeks at 7°C germinated after chilling and produced plantlets. Tetraploid forms were discovered within regenerated plants after chromosome counts of the root tips. A larger number of tetraploid plants were observed after treatment of embryogenic callus and formed embryoids with gamma-rays and colchicine. Plants were grown in the greenhouse and were field tested. Some plants generated from embryoids were more vigorous and produced berries with higher sugar levels.

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